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The association between erythrocyte internal viscosity, protein non-enzymatic glycosylation and erythrocyte membrane dynamic properties in juvenile diabetes mellitus

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Summary. The association of intracellular viscosity of red blood cells and the dynamic properties of erythrocyte membranes in children suffering from diabetes has been investigated by means of ESR spectroscopy. It has been revealed that the slight decrease in the ratio h_w/h_s of maleimide bound to membrane protein -SH groups of erythrocytes in diabetes may ensue from the enhanced membrane protein immobilization in the plane of lipid bilayer. These alterations were accompanied by a corresponding increase in the relative rotational correlation time (τ_c) of iodoacetamide spin label, thus suggesting that the conformational changes in membrane proteins may occur at both the intrinsic and more exposed thiol groups. The membranes of diabetic red blood cells were more glycosylated than those of relevant controls, and the extent of glycosylation was found to correlate significantly with h_{+1}/h_0 and τ_c ($r=-0.652$, $P<0.01$ and $r=0.609$, $P<0.01$). Further, the conformational alterations in erythrocyte membranes from diabetic subjects were accompanied by a significant increase in the mobility parameter (h_{+1}/h_0) of haemoglobin molecules in diabetic erythrocytes. The latter changes correlated well with the enhanced intracellular viscosity of diabetic red blood cells and the level of glycosylated haemoglobin.

We conclude that the alterations in membrane lipid–protein interactions together with the increased glycosylation-derived internal viscosity may consequently imply altered viscoelastic properties of erythrocyte membranes and, underlying the impaired deformability of red blood cells in the diabetic state, contribute to the development of late diabetic sequelae.

Keywords: diabetes mellitus, erythrocyte membrane, internal viscosity, ESR, non-enzymatic glycosylation, glycated haemoglobin, membrane proteins

Erythrocyte deformability, which has been defined as the ability of a cell to change its shape in response to an applied force, underlies the role of red blood cells in the microcirculation (Chien *et al.* 1987; McMillan *et al.* 1978). Under in-vivo conditions the whole cell deformability depends upon the cell surface area to volume index, the haemoglo-

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bin concentration, the intracellular viscosity and the viscoelasticity of the membrane including the cytoskeletal part (Clark 1989; Hochmuth & Waugh 1987). The evidence accumulated hitherto allows us to reason on a fairly well documented biochemical basis of erythrocyte deformability (Chasis & Shohet 1987). The mechanism underlying the high deformability of red blood cells has not been fully elucidated though an interaction of cytoskeletal proteins with the lipid bilayer has been suspected to be a critical factor.

Several impairments in blood rheology, such as reduced red blood cell deformability or enhanced plasma and whole blood viscosity, have been reported to accompany some diabetic complications, i.e. diabetic microvascular disease and diabetic angiopathy (Cecchin *et al.* 1987; MacRury *et al.* 1991; McMillan *et al.* 1978; McMillan 1976). Evidence accumulates supporting the idea that biochemical organization as well as dynamic properties of erythrocyte membranes may be considerably altered in the diabetic state. Reduced membrane lipid fluidity occasionally has been reported in diabetic patients and has been attributed to the altered membrane lipid content and the extent of non-enzymatic glycosylation of membrane proteins in diabetic erythrocytes (McMillan & Brooks 1982; Watala 1988; Watala *et al.* 1987; Watala & Kordacka 1987). It was deduced that erythrocyte internal viscosity is largely dependent upon the physicochemical properties of haemoglobin (Norton *et al.* 1981). Hyperglycaemia, which commonly accompanies the diabetic state, often leads to an increased glycosylation of haemoglobin molecules (Brownlee & Cerami 1981; Brownlee *et al.*, 1984; Wieland 1983). Although the role glycosylation plays in the impairment of haemoglobin functions *in vivo* is still disputable, it has been found that the non-enzymatic attachment of glucose *in vitro* may induce significant alterations in haemoglobin structure and conformation (Watala *et al.*, 1992). There are occasional reports that the viscosity of solutions of highly glycosylated haemoglobin is enhanced, and the increased internal viscosity of diabetic erythrocytes has been attributed to higher levels of glycosylated haemoglobin (Herrmann & Müller 1986; Maeda *et al.* 1982).

In the present study we made an attempt to correlate the extent of non-enzymatic glycosylation of membrane proteins and haemoglobin with red blood cell internal viscosity and altered conformation of haemoglobin and erythrocyte membrane proteins in juvenile diabetics.

Methods

Chemicals

Phenylmethanesulphonylfluoride (PMSF) was obtained from Merck (Darmstadt, FRG). Maleimido-TEMPO (4-maleimido-2,2,6,6-tetramethylpiperidine-1-oxyl, MSL), iodoacetamide spin label (ISL) and TEMPONE were purchased from Sigma Chemicals Co. (St Louis, MO). Glucose oxidase kit and affinity chromatography columns for test-combination of haemoglobin A_1 were from Boehringer Mannheim GmbH. All other chemicals were of AR grade from POCh (Gliwice, Poland). Water used for solution preparation and glassware washing was passed through a Millipore water purification unit (Millipore, Pty Ltd, England).

Subjects

Sixteen non-fasting diabetic patients (seven boys and nine girls, all type I; insulin-dependent) with a mean $\pm$ s.d. age of 11.9 ± 2.0 years (range 8–17) and 14 (11.4 ± 2.2 years, range 8–16) control subjects were studied. Mean body weights in control and diabetic subjects were respectively 43.8 ± 12.1 and 42.2 ± 9.3 kg. Mean duration of diabetes was 3.0 ± 1.8 years (range 0.3–8 years). All the diabetic subjects were treated with monocomponent insulins (0.83 ± 0.28 IU/kg body weight/day) and diet and were given three or four injections a day. Blood was collected from each diabetic subject and the paired control subject were

studied on the same day. These studies were approved by the Committee on the Ethics of Research in Human Experimentation at the Diabetological Hospital in Rabka and the Institute of Paediatrics, Medical School of Lódź.

Preparation of red blood cell membranes and haemoglobin

Human red blood cells washed four times with phosphate-buffered saline pH 7.4 were subjected to moderate haemolysis in Tris-HCl/EDTANa_2 buffer pH 7.0 according to Marchesi and Palade (1967). The isolated erythrocyte membranes were resuspended in ice-cold phosphate-buffered saline, used within a few hours thereafter, and kept at 4°C until used. The protein content in erythrocyte membrane suspensions was measured according to Lowry *et al.* (1951).

Haemoglobin was prepared according to Drabkin (1946) and purified on carboxymethylcellulose CM-32 (Tentori *et al.* 1965), and its concentration was measured by the method of Drabkin (1946).

Determination of HbA_{1c} and the extent of glycosylation

HbA_{1c} was determined using a glycoaffinity column (Allen *et al.* 1958) using Boehringer Mannheim GmbH kit. The normal range of HbA_{1c} is 4.5–7.5%. The extent of glycosylation was determined as described (Witztum *et al.* 1982). Aliquots of platelet membranes containing 100 μg protein were treated with equal volumes of 24 mmol/l ^{3}H-sodium borohydride (Amersham, England; 100 mCi/mmol) and incubated at 37°C for 2 h. After incubation, 3% bovine serum albumin was added to each sample and the protein precipitated with 10% TCA. The pellet was dissolved in 0.2 M NaOH and reprecipitated with TCA. This procedure was repeated four or five times until no further radioactivity could be detected in the supernatant. Radioactivity was measured in final pellet redissolved in 1 M NaOH. A standard curve was prepared using known concentrations of ^{3}H-sodium borohydride and the number of nmol ^{3}H-sodium borohydride bound per mg membrane protein was determined.

Spin labelling of erythrocyte membranes and haemoglobin

Erythrocyte membranes were labelled with maleimide spin label (2 μl of 100 mmol/l MSL solution per 1 ml of membrane suspension approx. 3 mg/ml protein) and then incubated for 1 h at room temperature, whereas the incubation with iodoacetamide spin label (2 μl of 100 mmol/l ISL solution per 1 ml of membrane suspension approx. 3 mg/ml) was performed overnight at 4°C. In the case of haemoglobin, the samples were labelled with maleimide spin label (2 μl of 100 mmol/l MSL ethanol solution per 1 ml of protein solution in dialysis buffer, approx. 3 mg/ml protein) and then incubated for 1 h at room temperature. The final ethanol concentration did not exceed 0.05% (v/v). The unbound spin label was removed by either extensive overnight dialysis against PBS at 4°C (haemoglobin) or four washings with PBS (3500 ***g***, 15 min). It was checked that no ESR signal could be detected in the dialysis buffer. Intracellular viscosity was determined according to Morse (1986). One μl of aqueous solution of TEMPONE (100 mmol/l) was introduced per 50 μl of an erythrocyte suspension (Ht = 50%) and incubated for 1 h at room temperature with gentle shaking. As the microviscosity of red blood cells is influenced by pH and osmolarity of the suspension medium (Morse & Warth 1990), the osmotic strength of the samples was maintained by using the appropriate concentrations of potassium ferricyanide in 5 mmol/l phosphate buffer, pH 7.4, as a line-broadening agent, prior to measurements. After incubation the suspension was centrifuged, the supernatant sucked off, and one volume of erythrocytes was suspended in five volumes of 80 mmol/l potassium ferricyanide in 5 mmol/l phosphate buffer, pH 7.4 to quench the ESR signal from the external

nitroxide. Under the conditions used, the ferricyanide was isosmotic with red blood cells. The efficiency of quenching was checked by scanning ESR spectra of TEMPONE in supernatant which showed a broadening typical for an erythrocyte-free system (Morse & Warth 1990).

In all the ESR spectra, the ordinate was represented as the amplitude of the ESR signal expressed in arbitrary units. ESR scannings were routinely recorded as the first derivatives of absorption spectra. The estimated ratios were calculated from the ESR graphs taking into account the relevant amplitudes measured as the heights of the respective peaks (expressed in metric units). ESR measurements were performed at ambient temperature (23 ± 1°C) in Bruker ESP 300E spectrometer.

Statistical analysis

Means ± s.d. are given. The data were elaborated by means of the two-tailed Student's *t*-test and non-parametric Mann–Whitney test. The normal distribution of data was checked by means of the Shapiro-Wilk's test (Zar 1984). A combined test for correlation coefficients was performed to assess relationships between the investigated parameters (Siegel & Castellan 1988).

Results

In order to monitor possible alterations in membrane protein conformation, two protein spin labels, MSL and ISL, were employed. Both spin labels are reactive toward thiol groups in proteins: whereas ISL is able to anchor preferentially at the surface of labelled protein, MSL may easily penetrate the interior of protein and also bind to the buried intrinsic -SH groups (Berliner 1983). As shown in Fig. 1, the spectrum of MSL attached to erythrocyte membranes consists of two dominant classes of spin label. The broad anisotropic spectrum arose from the subpopulation of label molecules embedded in strongly immobilized membrane environment; the narrower one, in turn, represents

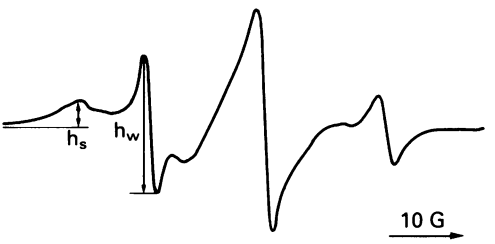

Fig. 1. ESR spectrum of 4-maleimido-2,2,6,6-tetramethylpiperidine-1-oxyl (MSL) attached to erythrocyte membrane ghosts; h_w and h_s correspond to weakly and strongly immobilized membrane MSL residues.

the subpopulation of weakly immobilized or more mobile MSL residues. The ratio of amplitudes of low-field peaks of weakly (h_w) to strongly (h_s) immobilized fraction of MSL is regarded as an indicator of the physical state of membrane proteins and, moreover, a sensitive measure of the conformational state of proteins (Berliner 1983). This ratio was reduced in diabetic patients (Table 2), thus suggesting that the thiol groups in membrane proteins may be more immobilized and have less freedom in the erythrocyte membranes originating from diabetic subjects. The observation was supported by the relevant increase in the values of the relative rotational correlation time (τ_c) of ISL, calculated from the heights and widths of spin label hyperfine lines according to the equation (Keith *et al.* 1970):

$$\tau_c = 6.5 \times 10^{-10} W_0 \{(h_0/h_{-1})^{1/2} - 1\} \quad (1)$$

where W_0, h_0 and h_{-1} are mid-field line width, mid-field line height and high-field line height, respectively (Fig. 2). The higher the values of τ_c, the slower the rotational mobility of ISL, which subsequently implies the stronger the immobilization of protein moieties. The values of τ_c estimated for diabetic and control subjects differed statistically (Table 2), indicating the considerable changes in the surroundings of surface thiol groups of erythrocyte membrane proteins. Furthermore, these alterations in membrane protein conformation were significantly correlated with the extent of glycosylation of

Table 1. Glycosylated haemoglobin (HbA_{1c}) and the extent of non-enzymatic glycosylation of proteins in erythrocyte membrane ghosts from diabetic and control subjects

	Diabetic (16)	Control (16)	*P*
HbA_{1c} (%)	9.74±2.3	4.8±0.4	<0.001
Glycosylation (nmol glucose/mg protein)	27.1±3.2	22.1±1.8	<0.001

Each value represents mean±1 s.d. Significance of differences calculated by Student's *t*-test.

Table 2. Iodoacetamide rotational correlation time (τ_c) and immobilization of maleimido-TEMPO (expressed as h_w/h_s ratio) in erythrocyte membranes from diabetic and control subjects

	Diabetic (15)	Control (15)	*P*
τ_c* (n.s.)	9.594±1.173	8.277±0.456	<0.01
h_w/h_s†	8.142±1.582	9.975±2.636	<0.03

Each value represents mean±1 s.d. Significance of differences calculated by * Student's *t*-test and † Mann–Whitney test.

membrane proteins but not with the amount of glycosylated haemoglobin (Table 4), although both the parameters were increased in diabetic subjects (Table 1). The rotational correlation time (τ_c) of TEMPONE was estimated according to equation (1) and the erythrocyte internal microviscosity was calculated from the ratio:

$$\eta_{er}/\eta_{water} = \tau_{c\,er}/\tau_{c\,water} \qquad (2)$$

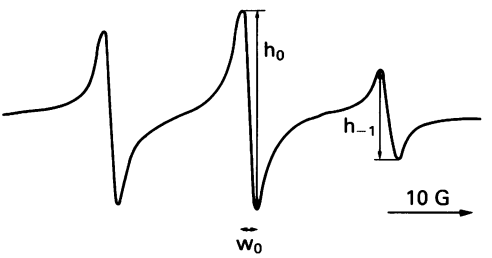

Fig. 2. ESR spectrum of iodoacetamide spin label (ISL) attached to -SH groups in erythrocyte membrane proteins; W_0, h_0 and h_{-1} are mid-field line width, mid-field line height and high-field line height, respectively.

where $\tau_{c\,er}$ and $\tau_{c\,water}$ are rotational correlation times for TEMPONE in erythrocytes and in bulk water, respectively, and η are internal microviscosities of red blood cells and viscosity of water (Morse 1986). The intracellular viscosity thus calculated was significantly greater in red blood cells from diabetic subjects than in controls (Table 3).

As shown in Fig. 3, the spectrum of MSL attached to haemoglobin consists of two

Table 3. Intracellular viscosity (η_c) (as monitored by TEMPONE) and immobilization of maleimido-TEMPO bound to haemoglobin (expressed as h_{+1}/h_0 ratio) in red blood cells from diabetic and control subjects

	Diabetic (16)	Control (16)	*P*
η_c	5.18±0.24	4.59±0.33	<0.01
h_{+1}/h_0	0.370±0.018	0.340±0.014	<0.03

Each value represents mean±1 s.d. Significance of differences calculated by Student's *t*-test.

Table 4. Correlation coefficients among various biophysical parameters and the glycosylation of haemoglobin (HbA_{1c}) and membrane proteins (NEG)

	h_w/h_s	τ_c	h_{+1}/h_0	η_c
NEG	−0.652	0.609	0.237	0.379
	(14)	(14)	(15)	(15)
	0.01	0.01		0.05
HbA_{1c}	−0.157	0.277	0.692	0.518
	(15)	(15)	(16)	(16)
			0.001	0.01
η_c	−0.478	0.572	0.711	
	(15)	(15)	(16)	*
	0.02	0.01	0.001	

Correlation coefficient (sample size) significance. When no value for P is shown, the correlation coefficient is not significant ($P>0.05$).

dominant classes of spin label and is typical for the spin label in strongly immobilized protein domains. The extreme left broad peak corresponds to the subpopulation of spin label molecules embedded in a strongly immobilized environment; as we evidenced in our previous study (Watala *et al.* 1992) it remains fairly stable and unchanged regardless of haemoglobin glycosylation. The right-adjacent peak, which fluctuated with the changes in haemoglobin glycosylation, represents the subpopulation of more mobile MSL residues. We used the ratio of amplitudes of the relatively mobile fraction in low-field line (peak height h_{+1}) to middle-field line (peak height h_0) in the MSL spectrum. The latter parameter, regarded as an indicator of the physical state of proteins (Watala *et al.* 1992) was significantly enhanced in haemoglobin prepared from diabetic erythrocytes (Table 3). This increase occurred together with the relevant elevation in overall erythrocyte internal viscosity in diabetic patients. Further, not only internal viscosity but also the values of the h_{+1}/h_0 parameter correlated significantly with the glycosylation of haemoglobin and moderately with the glycosylation of membrane proteins (Table 4).

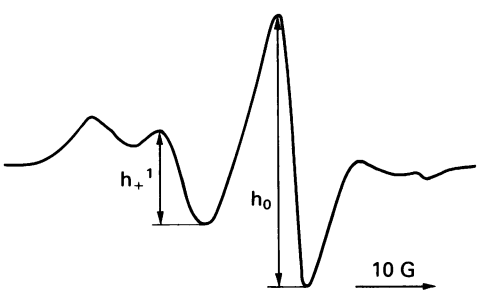

Fig. 3. ESR spectra of 4-maleimido-2,2,6,6-tetramethylpiperidine-1-oxyl (MSL) attached to -SH groups in human haemoglobin; h_{+1} and h_0 are the heights of low- and mid-field lines, respectively.

Discussion

It appears clear that in the course of diabetes the conformation of erythrocyte membrane proteins and haemoglobin may be considerably altered. Maleimide spin label employed in this study revealed a significantly increased immobilization of membrane proteins in diabetic erythrocytes, whereas the domains of thiol groups in haemoglobin from diabetic patients seemed to become more mobile. In fact, based on MSL spectrum in membranes, one might presume that the behaviour of spin label resembled very much the process of protein renaturation. Other-

wise, the corresponding 'expansion' in haemoglobin molecules in diabetes was typical for protein inactivation (Watala & Gwozdzinski 1992). The altered MSL spectrum was accompanied by a corresponding increase in the relative rotational correlation time of ISL. It should be borne in mind that the two spin labels have essentially different preferences when labelling protein-SH groups (Berliner 1983). Evidently, the surroundings of the surface thiol groups of membrane proteins in the erythrocytes from diabetic and control patients are not very much alike; hence the values of τ_c in more profoundly glycosylated diabetic membranes are higher, indicating that the supposed attachment of glucose might have induced membrane protein immobilization in the surface regions of the protein molecules. Likewise, upon the glycosylation of proteins in diabetic erythrocyte membranes, the intrinsic thiol groups become more immobilized, as revealed by the MSL h_w/h_s parameter. Such retardation and immobilization have been interpreted as reflecting aggregation or 'clustering' of the protein in the plane of the membrane (Clague & Cherry 1989) and are likely to influence the viscoelastic properties of cell membranes (Chien *et al.* 1987; Cecchin *et al.* 1987; Drasler *et al.* 1989). The immobilization of membrane proteins in diabetic erythrocytes was accompanied by a significant increase in the parameter h_{+1}/h_0, used herein as an indicator of the physical state and conformation of haemoglobin molecules. Along with these changes, the increase in the erythrocyte internal viscosity, which in turn may be accounted for by the glycosylation-derived structural alterations in haemoglobin molecules (Watala *et al.* 1992), is likely to bring about a reduced red blood cell deformability and consequently an impairment of blood rheology (Cecchin *et al.* 1987; McMillan *et al.* 1978). Our present results remain compatible with those obtained by Kern *et al.* (1989) and Bryszewska and Gwoździński (1989), who reported respectively the elevated whole blood viscosity in alloxan diabetic animals and the elevated erythrocyte internal viscosity in diabetic humans. In the study on human erythrocytes it was found that the process of glycosylation may affect the interaction between haemoglobin and red blood cell membranes (Bryszewska 1988). Featuring the role of glycosylated haemoglobin in the impairment of dynamic properties of erythrocyte membranes, it should be mentioned that various forms of haemoglobin were reported to bind to the negatively charged band 3 protein in erythrocyte membrane with different affinities. Non-enzymatic glycosylation, which alters protein conformation and diminishes its positive charge (Flückiger & Gallop 1984; Van Boekel 1991) may potentially affect the binding of haemoglobin with erythrocyte membrane (Chetrite & Cassoly 1985). The decreased membrane lipid fluidity (Watala *et al.* 1987; Watala & Kordacka 1987) and lowered deformability (Cecchin *et al.* 1987; McMillan *et al.* 1978) reported for diabetic erythrocytes might well result from disorders in haemoglobin–membrane interactions. In support of this idea, it needs to be emphasized that essentially the same mechanism was reported to occur in sickle erythrocytes (Evans & Mohandas 1987; Drasler *et al.* 1989). The results reported by Chien *et al.* (1987) support the notion that the rheological behaviour of sickle cells is an important link between the molecular events in haemoglobin S and the behaviour of the cell in the microcirculation. The effects of non-enzymatic glycosylation on the polymerization of HbS have been interpreted to be possibly due to modification by glycosylation of specific sites that are directly or indirectly involved in the intramolecular contacts (Abraham & Elseweidy 1986). The alterations of physicochemical properties of haemoglobin are intimately connected with its impaired functional characteristics (Samaja *et al.* 1982) and may contribute to the increased internal viscosity of red blood cells from diabetic subjects leading to reduced erythrocyte deformability (Cecchin *et al.* 1987) and handicapped oxygen release under tissue hypoxia in the diabetic state (McMillan 1976). Furthermore, the enhanced association of haemoglobin with erythrocyte membranes may impair

dynamic properties of membrane, and thus might underlie the handicapped red blood cell function (Hebbel 1991). In addition, the decreased levels of erythrocyte 2,3-DPG in diabetes (Barnes *et al.* 1977) may facilitate this enhanced association, thus further contributing to the lowered deformability of diabetic erythrocytes (Suzuki *et al.* 1990).

The simultaneous occurrence of the alterations in membrane lipid fluidity (Watala *et al.* 1987; Watala & Kordacka 1987) enters into the discussion of the possible variations in the structure and conformation of membrane proteins. Also, reorganization of lipid assemblies may imply alterations in lipid–protein interactions. The latter may produce local conformational changes in membrane proteins as a result of which lysine residues may be cooperatively modified in a high-glucose microenvironment.

A better understanding of the biochemical and biophysical mechanisms which supposedly underlie the intimate glucose-mediated impairment of membrane viscoelastic abilities and haemoglobin structure may further elucidate the molecular basis for diabetic microvascular disease and diabetic microangiopathy (Chien *et al.* 1987; MacRury *et al.* 1991; Maeda *et al.* 1982; McMillan 1976).

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