Alterations in the erythrocyte plasma membranes in patients with alcohol-induced liver cirrhosis – preliminary results

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Abstract

Introduction: Conformations of membrane proteins, membrane fluidity of erythrocytes in patients with AILC were studied with the use of electron paramagnetic resonance and spectrophotometric methods. The concentration of substances reacting with thiobarbituric acid was also determined. The aim of the study was to recognize the nature, level and causes of changes in the structure of erythrocytary membrane observed in erythrocytes of patients compared to erythrocytes from healthy controls.
Material and methods: Spin labels: MSL and ISL binding covalently to thiol groups of membrane cytoskeleton proteins were used to analyse modifications occurring in erythrocytary membrane proteins. Doxyl derivatives of fatty acids: 5-DS, 12-DS and 16-DS binding hydrophobically to erythrocytary membrane were used as spin labels for the analysis of erythrocyte membrane lipid fluidity.
Results: Modification of membrane cytoskeleton proteins and increase of membrane lipids fluidity were observed in erythrocytes of the investigated patients. An increase of the concentration of substances reacting with thiobarbituric acid was also confirmed in the erythrocytes of AILC patients.
Conclusions: Observed disorders in the structure of erythrocyte cytoskeleton proteins in patients, which might developed as a consequence of oxidative stress may be conformation changes in the structure of proteins which affect membrane cytoskeleton. The differences in the structure of membrane proteins could be associated with an increase in membrane lipids fluidity. Increased fluidity of erythrocyte membrane may be a result of disorders in protein-lipid interaction or membrane lipid peroxidation activity.

Key words: blood, peroxidation, cytoskeleton, fluidity.

Introduction

The differences in size and shape of erythrocytes, frequently observed in blood cell count in subjects hospitalized due to alcohol-induced liver cirrhosis (AILC), may affect their susceptibility to lysis. In patients with liver cirrhosis life span of erythrocytes was shorter by about 50-60% with coexisting osmotic fragility alerations [1-3]. Anemia, often observed in patients with AILC, is not the result of marrow erythrocytary line abnormality [4] but is most probably caused by intensified oxidation of erythrocyte membrane in the course of free radical processes. Increased

peroxidation and changes in plasma lipid composition in the liver of cirrhotic patients were observed [5-7], causing an increase in erythrocyte membrane fluidity, affecting cell metabolism [8-10]. Thus, the aim of the study was to determine structural changes in plasma membrane of subjects' erythrocytes with AILC compared to healthy controls.

Material and methods

Chemicals

Spin labels: 5-doxylstearic acid (5-DS), 12-doxylstearic acid (12-DS), 16-doxylstearic acids (16-DS), 4-maleimido-2,2,6,6-tetramethylpiperidine–1-oxyl (maleimide spin label, MSL) and 4-iodoacetamido-2,2,6,6-tetramethylpiperidine-1-oxyl (iodoacetamide spin label, ISL) were produced by Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals of analytical grade were purchased from POCh (Gliwice, Poland).

Material

Whole blood of 12 healthy controls (7 women, 5 men, aged 35-45 years) without any liver pathology or dysfunction of other organs and systems, the laboratory tests of subjects were within normal limits and of 12 patients (4 women, 8 men, aged 35-45 years) with clinically, including ultrasonography image of the liver and liver biopsy with tissue samples analysis, and laboratory diagnosed alcohol-induced liver cirrhosis, was the subject of investigations. Any other aetiologies of liver cirrhosis such as viral hepatitis, primary biliary cirrhosis were excluded as the first selection criteria for the patients. The investigated group is made up of the patients who are alcoholics and have been hospitalized in this clinic at various times as a result of alcohol overdose and alcohol disease complications.

There is an ethics committee approval for this research (approval No. RNN/49/06/KB date: 21.02.2006).

Preparation of erythrocytes

Whole blood was centrifuged and plasma as well as the leukocyte layer was removed. The remaining erythrocyte suspension was washed three times with ice-cold PBS solution of pH 7.4 and centrifuged at 3500 rpm for 10 min (Sigma 3k15). Then, a suspension of 50% hematocrit value was obtained for studied erythrocytes.

Isolation of erythrocyte membranes

Erythrocyte membranes were isolated using the modified method of Dodge [11]. Erythrocyte suspension was lysed using 20 mmol/l of phosphate buffer of pH 7.4 and centrifuged. Then, the procedure was repeated using the same solution of the concentration 10 mmol/l and 5 mmol/l in order to wash off the haemoglobin from the erythrocyte ghosts.

Physical state of membrane proteins

Conformation changes of membrane proteins were estimated with the electron paramagnetic resonance method using MSL and ISL spin labels. 2 µl of both labels in ethanol solution were added to 1 ml of erythrocyte membrane suspension and the samples were incubated at 4°C for 60 min. The excess of not bound label was washed out with ice-cold PBS solution of pH 7.4, until EPR signal disappeared. The ratio of h_w/h_s (h_w – the height of the amplitude of a line from the population of weakly immobilized spin label residue of label to the height of the amplitude of strongly immobilized component – h_s) was calculated from the obtained spectra of MSL attached to membrane proteins. In the case of ISL, the mobility of label bound to proteins was calculated as the relative rotation correlation time τ_c [12, 13] from the equation:

$$\tau_c = kw_0\left[\left(\frac{h_0}{h_{-1}}\right)^{-\frac{1}{2}} - 1\right]$$

Where: k – 6.5 10^{-10} , w_0 – width of middle line of EPR spectra, h_0 – height of middle line, h_1 – height of high-field line.

Analysis of erythrocyte membranes fluidity

Fluidity of erythrocyte lipid bilayer was estimated by electron paramagnetic resonance method using fatty acids doxyl derivatives (5, 12, 16-doxylstearic) as spin labels. 1 µl of each label in ethanol was added to 1 ml of erythrocyte suspension samples and then incubated for 30 min at room temperature. The ethanol concentration in erythrocyte suspensions did not exceed 0.1%. Spectra were obtained for all three labels and the ratio of the height of the low field line amplitude (h_{+1}) to the height of the middle line amplitude (h_0) was calculated [14, 15].

Determination of lipid peroxidation

The concentration of substances reacting with thiobarbituric acid was determined with Placer's [16] spectrophotometric method using Beckman DU 650 spectrophotometer. The mixture of thiobarbituric and trichloracetic acid was added to diluted erythrocyte hemolysate at the ratio of 3 : 1. The mixture was incubated in water bath at 100°C for 20 min, then after centrifugation the absorbance of supernatant was measured at wavelength 532 nm.

Calculating concentration of substances reacting with thiobarbituric acid was performed in relation to a blind test from the formula:

$$S = \frac{\Delta E \times 65.08}{gHb}$$

Where: S – concentration of substances reacting with thiobarbituric acid, ΔE – difference in absorbance of sample and blind test, gHb – haemoglobin mass in 100 ml of hemolysate.

EPR measurement

The measurements were performed at room temperature with Bruker ESP 300E (X-band) spectrometer operating at microwave frequency of 9.73 GHz using the following parameters of instrumental settings: centre field set at 3480 G, range 80 G with a 100 Hz modulation frequency and modulation amplitude 1.G at room temperature.

Statistical analysis

Statistical analysis included the calculation of means ± S.D. The significance of differences was estimated by Student *t*-test.

Results

The properties of erythrocyte plasma membrane from patients with alcohol-induced liver cirrhosis were investigated. Membrane proteins conformation, membrane lipids fluidity of erythrocytes were estimated with the use of spin labeling method in electron paramagnetic resonance spectroscopy and by spectrophotometric method. Two spin labels, maleimide (MSL) and iodoacetemide (ISL) covalently bound to protein thiol groups, mainly of the spectrin/actin complex were applied in the studies of physical state of membrane proteins. The ratio of hw/hs was calculated from EPR spectra of MSL attached to membrane proteins. This parameter is a sensitive indicator of protein physical state. Calculated ratio of h_w/h_s in the investigated group was 0.27 compared to 0.37 in the group of healthy controls, (statistically significant differences) (Figure 1).

Changes in the physical state of membrane proteins were observed also in the case of iodoacetemide (ISL) label. Figure 2 demonstrates calculated time of rotation correlation of the label bound to membrane proteins, which in the investigated group equals 3.77 ns compared to 6,99 ns in group of healthy controls, results are statistically significant.

Three spin labeled fatty acids 5-DS, 12-DS and 16-DS, having paramagnetic group at different carbon atoms of hydrocarbon chain, were used for the estimation of lipid membrane fluidity. The ratio of the height of low field amplitude to the height of the spectrum middle line (h_{+1}/h_0) was calculated from spectra of labelled fatty acids incorporated to lipid membrane. Calculated ratio h_{+1}/h_0 for the label 5-DS in the investigated group was 0.35 compared to 0.27 in the group of healthy controls, (statistically significant increase). For the label 12-DS obtained result of the ratio h_{+1}/h_0 in patients group was 0.46 compared to 0.39 in the control group, (statistically significant increase). In the case of 16-DS label the differences were not statistically significant (Figure 3).

The level of lipid peroxidation in erythrocytes from patients with alcohol-induced liver cirrhosis using thiobarbituric acid was determined. This indicator is used the most frequently in identification of oxidative stress. Erythrocytes of patients manifested higher concentration of substances reacting with thiobarbituric acid, result in the invesigated group was 0.32 µmol/gHb

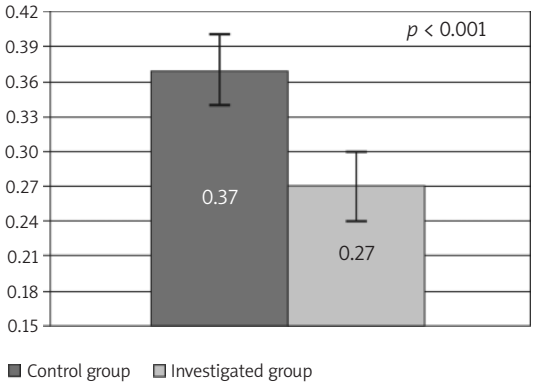

Figure 1. Changes in hw/hs ratio of maleimide label (MSL) bound to control erythrocyte membranes and to erythrocyte membranes from alcohol-induced liver cirrhosis. Means with standard deviations are presented (n = 12, p < 0.001)

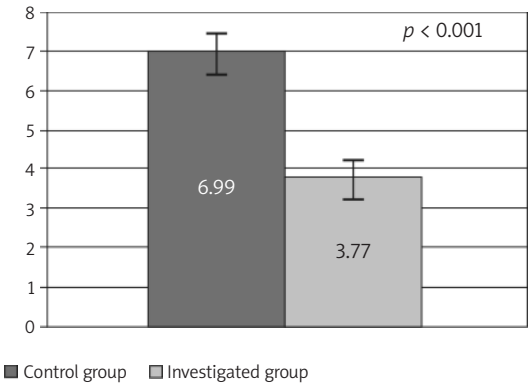

Figure 2. Differences in the relative rotational correlation time (τ_c) of iodoacetamide label bound to membrane erythrocyte proteins of patients with AILC compared to the control group. Means with standard deviations are presented (n = 12, p < 0.001)

Table I. Selected results of blood tests of patients with AILC (investigated group)

Patient	ALT	AST	ALP	GGTP	Bilirubin [mg/dl]	INR	Albumin [g/dl]
1. ♂	187	297	300	890	11.18	1.15	2.31
2. ♂	11	59	167	56	3.65	1.07	2.33
3. ♀	100	84	228	994	3.6	1.21	2.8
4. ♂	45	177	229	394	6.52	1.77	3.12
5. ♂	190	71	158	156	22.4	1.91	3.44
6. ♀	38	149	146	392	9.08	1.92	3.34
7. ♀	13	76	243	647	15.63	1.02	2.86
8. ♂	75	105	173	811	16.51	1.14	2.67
9. ♂	42	76	258	252	6.26	1.16	2.64
10. ♂	29	114	313	265	12.36	1.21	2.59
11. ♀	106	240	550	400	10.04	0.82	2.66
12. ♂	18	90	208	573	5.5	1.73	2. 91
Patient	**WBC**	**RBC**	**HGB**	**HCT**	**MCV**	**MCH**	**Child-Pugh Score**
1. ♂	5.25	3.53	11.9	35.5	100.4	33.6	C
2. ♂	16.15	3.26	8.5	27.1	83.2	26	C
3. ♀	4.56	3.56	12.8	37.6	105.6	36.1	C
4. ♂	8.95	2.62	9.8	27	102.8	37.4	C
5. ♂	7.1	4.2	12	37	104.3	34	C
6. ♀	13	2.31	10.5	28.5	123.4	35.6	C
7. ♀	34.23	2.64	9.5	28.5	108.1	36.5	C
8. ♂	7.88	3.01	10.5	31.5	104.9	34.8	C
9. ♂	3.13	3.05	9.5	28.8	94.5	30.1	C
10. ♂	23.21	3.35	11.3	34.6	103.2	33.6	C
11. ♀	6.8	3.8	13.2	38.6	101.6	34.9	C
12. ♂	7.8	2.86	9.9	30.1	105.2	34.6	C

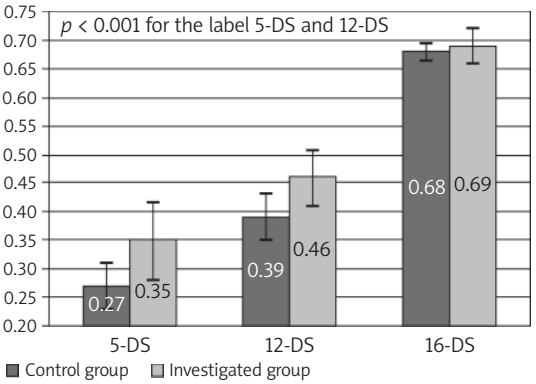

Figure 3. The ratio of h_{+1}/h_0 for 5-DS, 12-DS and 16-DS labels incorporated into erythrocyte membrane lipids of controls and patients with AILC. Means and standard deviations are presented (n = 12, for the label 5-DS and 12-DS $p < 0.01$)

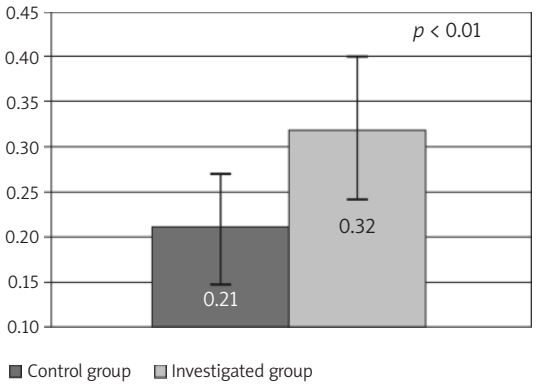

Figure 4. Concentration of substances reacting with thiobarbituric acid in erythrocytes of controls and patients with AILC (n = 12, $p < 0.01$)

compared to 0.21 in the group of healthy controls (differences statistically significant) (Figure 4).

Discussion

Liver cirrhosis as a result of frequent and long term alcohol intake or viral hepatitis can lead to chronic liver failure with associated alterations in blood cells or hepatocellular carcinoma evolving on the basis of chronic hepatitis [17]. It has been shown that erythrocytes from patients with AILC have different count, size and shape, that they are more susceptible to lysis than normal ones and that their life span is much shorter, about 50-60% and is correlated with higher osmotic fragility [1-3].

These findings formed the base for study of erythrocyte plasma membrane properties in this disease. Conformation of membrane cytoskeleton proteins, physical state of peripheral proteins and membrane lipid fluidity were determined using spin labelling method in EPR spectroscopy. These parameters can affect the erythrocyte shape and size and can also reflect osmotic fragility of erythrocytes [3].

MSL and ISL spin labels were used in the study on membrane proteins conformation, mainly of spectrin/actin complex. EPR spectra of the labels point to obvious disorders in the structure of erythrocyte cytoskeleton proteins in patients, which might have developed as a consequence of oxidative stress [18, 19]. These could result in conformation changes in the structure of proteins or/and their thiol groups oxidation, as they are the most sensitive to oxidation [20, 21]. Modifications in spectrin/actin complex may affect membrane cytoskeleton structure.

The differences in the structure of membrane proteins observed in the group of patients could be associated with an increase in membrane lipids fluidity at the depth of 5^{th} atom of carbon of fatty acid hydrocarbon chain, which points to modification of polar heads of phospholipids in the surface areas of cell membrane. Similar changes were observed at the depth of 12^{th} carbon atom of spin probe. Increased fluidity of erythrocyte membrane in the above mentioned areas may be a result of disorders in protein-lipid interaction or membrane lipid peroxidation activity [18, 20].

Increase of fluidity of erythrocyte membrane lipids was also observed in model studies with t-butylhydroperoxide which initiated the process of peroxidation [22]. Elevated concentration of substances reacting with thiobarbituric acid confirms of the occurrence of oxidative stress in the group of patients. It is very probable that the increase of lipids fluidity in the membrane could be the consequence of their peroxidation.

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