Current Topics in Biophysics 2010, 33 (suppl A), 177-181

ALTERATIONS IN HUMAN RED BLOOD CELL MEMBRANE PROPERTIES IN ALZHEIMER DISEASE PATIENTS

ANNA PIENIĄŻEK[1], ROBERT HAJDUK[2], MICHAL POPIŃSKI[2], ELŻBIETA POZIOMSKA-PIĄTKOWSKA[2], KRZYSZTOF GWOŹDZIŃSKI[3*]

[1]Department of Thermobiology, University of Lodz,
[2]Department of Biomedical Bases of Rehabilitation, Medical University of Lodz, Poland
[3]Department of Molecular Biophysics, University of Lodz,

Received September 10, 2010; accepted October 14, 2010; published online November 20, 2010.

Alterations in erythrocytes from Alzheimer disease patients were examined using EPR spectroscopy. Three spin-labeled fatty acids were applied to measure lipid membrane fluidity. A decrease of membrane fluidity was found in the deeper region of lipid bilayer of erythrocytes membrane, as indicated by 12-doxylstearic acid assay. The physical state of erythrocyte membrane proteins was estimated using 4-maleimido-2,2,6,6-tetramethylpiperidine-1-oxyl (MSL) and 4-iodoacetamido-2,2,6,6-tetramethylpiperidine-1-oxyl (ISL). An increase in the ratio of weakly to strongly immobilized fractions of MSL and an increase in mobility of ISL attached to the membrane in Alzheimer disease patients were found. A decrease in lipid membrane fluidity may be a consequence of lipid peroxidation or/and alterations in lipid protein interaction. The increase in membrane protein mobility can be a result of protein oxidation. It is possible that changes in the plasma membrane may be a result of membrane components oxidation or β-amyloid peptide interaction with erythrocytes.

INTRODUCTION

The central nervous system is particularly vulnerable to damage by oxidative stress because of the highest oxygen consumption in brain tissue. Brain tissue is rich in polyunsaturated fatty acids, which are sensitive to oxidation (Coyle & Puttfarcken, 1993). There are several observations suggesting connection of Alzheimer disease (AD) with oxidative stress and numerous evidence of oxidation of lipids, proteins and DNA, indicating the participation of free radicals in the etiology process (Markesbery, 1997). Other arguments that point to the occurrence of oxidative stress regard the higher content of metals such as iron, copper and aluminum in the brain of AD patients. Iron and copper are catalysts in Fenton and/or Haber-Weiss type reactions, both of which produce the highly reactive hydroxyl radical. It has been shown that aluminum can also be a potential catalyst in reactions leading to oxidative stress. Alzheimer patients have altered antioxidant systems in plasma and in erythrocytes (RBC). A higher level of malondialdehyde (MDA), an indicator of oxidative stress, was found in plasma (Burdel-Marchasson, Delmas-Beauviex, Peuchant, Richard-Harston, Decamps, Reignier, Emeriau & Rainfray, 2001). The same authors described a diminished content of α-tocoferol, retinol and uric acid in the plasma of AD patients, but a similar level of vitamins as well as superoxide dismutase and glutatione peroxidase enzyme activities was found in AD and normal red blood cells. On the other hand, higher SOD activity was observed by Delibas et al. (Delibas, Ozcankaya & Altuntas, 2002) and Rossi et al. (Rossi, Squitti, Pasqualetti, Marchese, Casetta, Forastiere, Rotillo, Rossini & Finazzi-Agro & 2002). A contrary result (a decrease in SOD activity) was described by Rinaldi et al. (Rinaldi, Polidori, Metastasio, Mariani, Matiolli, Cherubini, Catani, Cecchetti, Senin & Mecocci, 2003).

In Alzheimer disease, an extracellular accumulation of β-amyloid peptide takes place. β-amyloid was found in the cerebrospinal fluid and in the plasma of AD patients. The interaction between peptide particles and red blood cells was found and it has been suggested that red blood cells can be altered in AD patients.

The aim of this study was the determination of properties of red blood cell plasma membrane in

* Corresponding author: kgwozdz@biol.uni.lodz.pl

Alzheimer disease. For elucidation of these properties, lipid membrane fluidity, physical state of membrane proteins and osmotic fragility of red blood cells were examined applying spin labeling and spectrophotometric methods.

MATERIAL AND MATHODS

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-tetramethylpiperidi-ne-1-oxyl (iodoacetamide spin label, ISL) were purchased from Sigma-Aldrich (Poznan, Poland). All other chemicals of analytical grade were purchased from POCh (Gliwice, Poland).

Isolation of red blood cells and RBC ghosts

Human blood was collected from ten patients (in the age range between 60 and 85) diagnosed with Alzheimer disease according to the DSM-IV criteria for dementia in the Clinic of Psychology of the Medical Military Academy in Krapkowice. Control human blood was taken from ten healthy donors (in the age range between 56 and 81) without Alzheimer symptoms, attending the outpatient clinic of the local hospital. Blood was anticoagulated with heparin and stored before experiments for 24 hours at 4°C. Red blood cells were isolated by centrifugation and subsequently washed three times with cold phosphate buffered saline (PBS) pH 7.4 at 4°C. The packed cells were suspended in PBS at a hematocrit of 50%.

The RBC ghosts were prepared by the modified method of Dodge et al. (Dodge, Mitchell & Hanahan, 1963) including hypotonic lysis using 20 mmol/l sodium phosphate buffer, pH 7.4 at 4°C. The ghosts were successively washed with 20 mmol/L, 10 mmol/L and 5 mmol/L phosphate buffer, pH 7.4.

Spin labeling of red blood cells and RBC ghosts

Erythrocytes were labeled with 5-doxylstearic acid, 12-doxylstearic acid and 16-doxylstearic acid by the introduction of stock solution of the spin label in ethanol into red blood cell suspension, and incubated for 0.5 h at room temperature. The final ethanol concentration in erythrocyte suspension did not exceed 0.05% (v/v).

Isolated erythrocyte ghosts were labeled with maleimide or iodoacetamide spin label using 2 μl of ethanol solution of 0.1 mol/L of appropriate spin label per 1 ml of ghost suspension (approx. 3 mg/ml of protein) and incubated for 1 h at 4°C. The unbound spin label was removed by several washings with cold phosphate buffer until the EPR signal in supernatant disappeared. All procedures were performed at 4°C.

Osmotic fragility measurements

For osmotic fragility measurements, erythrocyte suspensions were added to solutions containing increasing concentrations of sodium chloride. The absorbance of supernatant was measured in a Pharmacia-LKB spectrophotometer at 575 nm. The extent of hemolysis was calculated from the equation (1):

$$H = \frac{A_x - A_c}{A_{100} - A_c}, \qquad (1)$$

where H denotes the extent of hemolysis, A_x, absorbance of sample, A_c, absorbance of control, and A_{100} absorbance after complete hemolysis.

EPR measurements

EPR measurements were performed in a Bruker ESP-300E (X-band) spectrometer operating at microwave frequency of 9.73 GHz at ambient temperature. The typical instrumental parameters were as follows: center field set at 3480 G, range 80 G with a 100 Hz modulation frequency and modulation amplitude 1.01 mT. All measurements were made at room temperature. The analysis of ESR spectra of the applied spin labels was described in detail elsewhere (Morrisett, Pownall, Plumlee, Smith, Zahner, Esfahani & Wakil, 1975; Stuhn-Sekalec & Stanacev, 1978).

Statistical analysis

Statistical analysis included the calculation of means ± S.D. The significance of differences was estimated using Student's t-test.

RESULTS

Three doxyl derivatives of fatty acids: 5-doxylstearic acid, 12-doxylstearic acid and 16-doxylstearic acid were applied for determination of lipid membrane fluidity. In the case of these labels, the paramagnetic reporter group is located at different depths of the lipid bilayer and gives the information on phospholipid fatty acyl mobility. For 5-DS the order

parameter S was calculated from equation (2):

$$S = \frac{T_{II} - T_{\perp}}{T_{zz} - T_{xx}} \cdot \frac{a_N}{a'_N} \quad (2)$$

where T_{II} and $T_{\perp}$ are hyperfine splitting constants for the magnetic field parallel and perpendicular to the bilayer normal, respectively; T_{zz} and T_{xx} are hyperfine splitting constants for nitroxide in the host crystal [T_{zz} = 32,4 G, T_{xx} = 6,1 G]; while a_N and a'_N are the isotropic hyperfine coupling constants for nitroxide in the membrane and crystal state, respectively, i.e.

$$a_N = \frac{1}{3}(T_{zz} + 2T_{xx}) \quad \text{and} \quad a'_N = \frac{1}{3}(T_{II} + 2T_{\perp})$$

(Seelig, 1970).

Table 1 shows a slight decrease in the order parameter of 5-DS incorporated into control and AD patient erythrocytes. For 12-DS, the ratio of h_{+1}/h_0 (where h_{+1} and h_0 are the heights of low-field line and middle-field line of the spectra, respectively) was determined as a semi-quantitative measure of acyl chain flexibility corresponding to lipid bilayer fluidity (Morrisett et al., 1975; Stuhn-Sekalec & Stanacev, 1978). A significant decrease in the h_{+1}/h_0 ratio was observed for 12-DS in AD red blood cells (Table 1). Changes in h_{+1}/h_0 ratio reflect a decline in membrane lipid fluidity. For 16-DS, the relative correlation time was calculated from the Kivelson equation (3) (Kivelson, 1960):

$$\tau_c = k \cdot w_0 \left[\left(\frac{h_0}{h_{-1}} \right)^{-\frac{1}{2}} - 1 \right] \quad (3)$$

where h_0 and w_0 are height and width of the mid-field line and h_{-1} is height of the high-field line, respectively.

Table 1. The order parameter, the ratio h_{+1}/h_0 and the rotational correlation time of 5-DS, 12-DS and 16-DS, respectively, in control and Alzheimer's RBC.

Spin label and parameter	Control n = 9	Alzheimer n=10
5-DS S	0.761 ± 0.007	0.748 ± 0.007
12-DS h_{+1}/h_0	0.610 ± 0.036	0.508 ± 0.055*
16-DS τ_c [s $\times 10^{-10}$]	23.73 ± 2.03	22.09 ± 1.99

*significant difference, $p < 0.005$

Conformation of membrane proteins was estimated using two covalently bound spin labels, MSL and ISL, which react mainly with the thiol groups of proteins in physiological pH (Berliner, 1983). The EPR spectrum of MSL attached to intact human erythrocyte ghosts is characterized by two components: weakly immobilized (narrow-line, h_w) and strongly immobilized (broad-line, h_s). The h_w/h_s ratio is commonly used in evaluation of the physical state of membrane proteins (Butterfield, 1982; Fung & Simpson, 1979; Fung, 1983). Application of MSL spin label shows an increase in the h_w/h_s ratio, which reflects changes in the conformation of membrane proteins, mainly in the cytoskeleton, in AD erythrocytes. However, the difference was not statistically significant (Table 2).

Table 2. The ratio of h_w/h_s and the ratio of h_{+1}/h_0 of MSL and ISL, respectively, in control and Alzheimer disease RBC.

Spin label and parameter	Control n = 9	Alzheimer n=10
MSL h_w/h_s	3.43 ± 0.12	3.81 ± 0.40
ISL h_{+1}/h_0	0.795 ± 0.024	0.853 ± 0.019*
Osmotic fragility $C_{(50)}$	76.06 ± 2.22	77.67 ± 1.94

*significant difference, $p < 0.0002$

Another spin label, ISL, yields a simple triplet spectrum when bound to erythrocyte membrane (ghosts). For detecting possible changes in membrane proteins, the ratio of h_{+1}/h_0 was calculated.

Table 2 shows an alteration in the ratio of h_{+1}/h_0 of ISL spin label attached to membrane proteins in AD. The higher values of this ratio reflect a significant increase in the mobility of spin labeled proteins in the membrane. Both covalently bound spin label show arise in the mobility of membrane proteins which can indicate their conformational changes.

DISCUSSION

Low activities of antioxidant enzymes as well as low level of low-molecular-weight antioxidants may lead to damage of red blood cells. There are a number of experimental proofs of age-related elevation in the level of oxidative stress in AD patients. A higher level of lipid peroxidation products in red blood cells has been shown (Ajmani, Metter, Jaykumar, Ingram, Spanger, Abugo & Rifkind, 2000; Gerasimov, Goloshchapov & Burlakova, 2009) as well as lower

level of total antioxidant status (Vaisi-Raygani, Rahimi, Zahraie, Noroozian & Pourmotabbed, 2007).

In this paper the alterations in plasma membrane properties of AD erythrocytes were determined by measuring lipid fluidity, conformation state of membrane proteins and osmotic fragility of erythrocytes.

Using 5-doxylstearic acid for determination of membrane lipid fluidity, we did not observe any significant increase in membrane lipid motion near the membrane surface, close to the polar region. Similar results were found for isolated red blood cell membranes (Butterfield & Markesbery, 1980). On the other hand, deeper regions of the lipid bilayer of AD erythrocytes show lower mobility of lipid hydrocarbon chains, as indicated by 12-doxylstearic acid assay. Increase in membrane lipid fluidity of erythrocytes was also observed in pathologies connected with oxidative stress like chronic renal failure, diabetes mellitus and other (McGrath, Douglas, McClean, Brown, Doherty, Johnson & Archbold, 1995; Bryszewska, Watała & Torzewska, 1986). It has also been reported using viscosimetric methods that red blood cell rigidity increases with age (Ajmani et al., 2000). Other study of erythrocyte membrane in patients with Alzheimer's disease show changes in their lipid fluidity in comparison to control erythrocytes (Gerasimov et al., 2009). Our results are in agreement with these findings.

In the case of maleimide spin label attached to membrane proteins of erythrocytes, an increase in the mobility of spin label residue was observed. It has been shown that this label is attached to the internal surface of membrane, mainly to spectrin-actin complex (Fung & Simpson, 1979; Fung, 1983). An increase in the mobility of spin label residue was found in AD erythrocytes. An (increase in the amount of MSL spin label residue attached to red blood cell membranes was also reported (Butterfield & Markesbery, 1980). The enhanced mobility of MSL spin label bound to membrane proteins may indicate oxidative damage of this complex or its dissociation. The increase in mobility of MSL was also observed in erythrocytes exposed to oxidative damage as well as in chronic renal failure patients (Janicka, Gwozdzinski, Weclewska, Luciak & Pawlicki, 1996; Brzeszczyńska & Gwoździński, 1999). Tendency to increase the mobility of spin label residues (not statistically significant) was found for ISL in AD erythrocytes. Despite of changes in membrane properties we did not find any changes in osmotic fragility of AD erythrocytes. The alteration in plasma membrane properties can affect deformability of erythrocytes and can lead to perturbation in microcirculation in AD patients. The results obtained by Mohanty et al. shows RBC shape/morphology changes in AD subjects (Mohanty, Shukla, Williamson, Launer, Saxena & Rifkind, 2010). The authors suggest that changes in RBC morphology may be a consequence of cytoskeletal proteins abnormalities. On the other hand, the life span of red blood cells in AD is shorter than in the healthy subject. These results confirm that changes in red blood cell membranes can lead to elimination of altered cells from the blood. Other researchers suggest that abnormalities in the structure of erythrocyte membrane may play a major role in the development of Alzheimer's disease (Gerasimov et al., 2009).

The extracellular accumulation of β-amyloid peptide is characteristic for Alzheimer disease. This peptide present in plasma can react with red blood cells (Kuo, Kokjochn, Kalback, Luehrs, Galasko, Chevallier, Koo, Emmerling & Roher, 2000; Jajakumar, Kusiak, Chrest, Demehin, Murali, Wersto, Nagababu, Ravi & Rifkind, 2003). It is possible that changes in plasma membranes of red blood cells may be a consequence of both oxidative damage and membrane-β-amyloid interactions.

REFERENCES

Ajmani R.S., Metter E.J., Jaykumar R., Ingram D.K., Spanger E.L., Abugo O.O., Rifkind J.M. (2000). Hemodynamic changes during aging associated with cerebral blood flow and impaired cognitive function. Neurobiol. Aging **21**. 257–269.

Berliner L.J., (1983). The spin label approach to labeling membrane protein sulfhydryl groups. Ann. NY Acad. Sci. **414**. 153–161.

Bryszewska M., Watała C., Torzewska W. (1986). Changes in fluidity and composition of erythrocyte membranes and in composition of plasma lipids in type I diabetes. Brit. J. Hematol. **62**. 111–116.

Brzeszczyńska J., Gwoździński K. (1999). t-Butylhydroperoxide-induced alterations in erythrocyte components. Curr. Top. Biophys. **23**. 113–118.

Burdel-Marchasson I., Delmas-Beauviex M.C., Peuchant E., Richard-Harston S., Decamps A., Reignier B., Emeriau J.P., Rainfray M. (2001). Antioxidant defences and oxidative stress markers in erythrocytes and plasma from normaly nourished elderly Alzheimer patients. Age Ageing **30**. 235–241.

Butterfield D.A., (1982). Spin labeling in disease, in: (L.J. Berliner, J. Reuben (Eds.), Biological magnetic resonance. Plenum Publishing Corp., New York, 1–78.

Butterfield D.A., Markesbery W.R. (1980). Specificity of biophysical and biochemical alterations in erythrocyte

membranes in neurological disorders. J. Neurol. Sci. **47**. 261–271.

Coyle J.T., Puttfarcken P. (1993). Oxidative stress, glutamate, and neurodegenerative disorders. Science **262**, 689 –695.

Delibas N., Ozcankaya R., Altuntas I. (2002). Clinical importance of erythrocyte malondialdehyde levels as a marker for cognitive deterioration in patients with dementia of Alzheimer type: a repeated study in 5-yr interval. Clin. Biochem. **35**. 137–141.

Dodge J.T., Mitchell C., Hanahan D.J., (1963). The preparation and chemical characteristic of hemoglobin-free ghost of human erythrocytes. Arch. Biochem. Biophys. **100**. 119–128.

Fung L.W., (1983). Analysis of spin labeled erythrocyte membrane. Ann. NY Acad. Sci. **414**. 162-167.

Fung L.W., Simpson M.J. (1979). Topology of a protein spin label in erythrocyte membranes. FEBS Lett. **108**. 269–273.

Gerasimov N. Yu., Goloshchapov A. N., Burlakova E. B. (2009). Structural State of Erythrocyte Membranes in a Human with Alzheimer's Disease. J. Phys. Chem. B. **3**. 652–655.

Jajakumar R., Kusiak J.W., Chrest S.J., Demehin A.A., Murali J., Wersto R.P., Nagababu E., Ravi L., Rifkind J.M. (2003). Red cell perturbation by amyloid beta-protein. Biochim. Biophys. Acta **1622**. 20–28.

Janicka M., Gwozdzinski K., Weclewska U., Luciak M., Pawlicki L. (1996). Role oxygen free radicals in erythrocytes damage in chronic renal failure. Curr. Top. Biophys. **20**. 72–75.

Kivelson D., (1960). Theory of ESR linewidth of free radicals. J. Chem. Phys. **33**. 1094–1106.

Kuo Y.M., Kokjochn T.A., Kalback W., Luehrs D., Galasko D.R., Chevallier N., Koo E.H., Emmerling M.R., Roher A.E. (2000). Amyloid-beta peptides interact with plasma proteins and erythrocyte: implications for their quantitation in plasma. Biochem. Biophys. Res. Commun. **268**. 750–756.

Markesbery W.R. (1997). Oxidative stress hypothesis in Alzheimer's disease. Free Radic. Biol. Med. **23**. 134 –147.

McGrath L.T., Douglas A.F., McClean E., Brown J.H., Doherty C.C., Johnson G.D., Archbold G.P.R. (1995). Oxidative stress and erythrocyte membrane fluidity in patients on undergoing dialysis. Clin. Chim. Acta **235**. 179–188.

Mohanty J.G., Shukla H.D., Williamson J.D., Launer L.J., Saxena S., Rifkind J.M. (2010). Alterations in the red blood cell membrane proteome in alzheimer's subjects reflect disease-related changes and provide insight into altered cell morphology. Proteome Science **8**. 11

Morrisett J.D., Pownall H.J., Plumlee R.T., Smith L.C., Zahner Z.E., Esfahani M., Wakil S.J. (1975). Multiple thermotropic phase transition in Escherichia coli membranes and membrane lipids. J. Biol. Chem. **250**. 6969–6976.

Rinaldi P., Polidori M.C., Metastasio A., Mariani E., Matiolli P., Cherubini A., Catani M., Cecchetti R., Senin U., Mecocci P. (2003). Plasma antioxidants are similarly depleted in mild and cognitive impairment and in Alzheimer disease. Neurobiol. Aging **24**. 915–919.

Rossi L., Squitti R., Pasqualetti P., Marchese E., Casetta E., Forastiere E., Rotillo G., Rossini P.M., Finazzi-Agro A. (2002). Red blood cell copper, zinc superoxide dismutase activity is higher in Alzheimer's disease and is decreased by d-penicillamine. Neurosci. Lett. **30**. 137 –140.

Seelig J., (1970). Spin label studies of oriented smectic liquid crystals. J. Am. Chem. Soc. **92**. 3881–3887.

Stuhn-Sekalec L., Stanacev N.Z., (1978). Temperature-induced changes spin-labeled radioactive lipids in isolated guinea pig liver microsomal membranes before and, in mitochondrial membranes, after translocation. Can. J. Biochem. **56**. 722–728.

Vaisi-Raygani A., Rahimi Z., Zahraie M., Noroozian M., Pourmotabbed A. (2007). Enzymatic and non-enzymatic antioxidant defense in alzheimer's disease. Acta. Med. Iran. **45**. 271–276.