Biochimica et Biophysica Acta, 1022 (1990) 260–264
Elsevier

BBAMEM 74736

Carriers of ataxia-telangiectasia gene display additional protein fraction and changes in the environment of SH groups in erythrocyte membrane

Maria Rybczynska [1], Andrzej L. Pawlak [2], Stanislaw K. Hoffmann [3] and Roman Ignatowicz [4]

[1] *Department of Biochemistry, Medical Academy, Poznan,* [2] *Institute of Human Genetics, Polish Academy of Sciences, Poznan,* [3] *Institute of Molecular Physics, Polish Academy of Sciences, Poznan,* [4] *Hospital-Monument Child Health Center, Warszawa (Poland)*

(Received 25 October 1989)

Key words: Ataxia-telangiectasia gene; Erythrocyte membrane; ESR; (Human)

Additional protein fraction migrating slower than spectrin has been detected in erythrocyte membranes from an ataxia-telangiectasia (A-T) patient and from his mother (A-T heterozygote). In erythrocyte membranes labelled with maleimide spin label changes in signal of the weakly immobilized spin label as related to that of strongly immobilized one (w/s) were noted. In comparison to age-matched control groups the values of w/s were lower in A-T heterozygotes (ten persons) and higher in A-T homozygotes (four persons). In control persons the values of w/s increased with age, whereas in families with A-T no significant differences in this parameter were noted between children and parents. The presence of additional protein fraction in erythrocytes membranes of A-T patient and A-T heterozygote indicates that these phenotypes can be differentiated from the healthy control persons for the first time on the basis of changes detected in the erythrocytes. This change in erythrocyte membrane may explain the decrease in the w/s parameter of electron spin resonance in A-T heterozygotes. On the other hand increased values of w/s in A-T patients may be caused by disease process.

Introduction

Ataxia-telangiectasia (A-T) is a monogenically inherited autosomal recessive disorder (Mc Kussick code number 20890) and its gene has recently been mapped to chromosome 11q22-23 [1]. It is characterized by clinical hypersensitivity to therapeutic X-rays, chromosomal instability and many neurological and immunological abnormalities. Patients rarely live longer than 20 years and in 20% of cases die of cancer [2]. The disease is heterogenous and at least four complementation groups were differentiated on basis of expression on cellular level [3]. Radiosensitivity in this syndrome is also expressed at the cellular level.

Homozygotes of A-T occur with a frequency of 1 per 40 000 births, but heterozygotes make up for between 0.6 and 2.8 percent of the population [4]. They display a 3-fold increased risk of cancer in general [5] and cardiovascular disease [6], but a 6-fold increased risk of mammary gland cancer and a 12-fold increased risk of leukemia [5].

Many of the biological effects of ionizing radiation are induced by changes in the membranes, which are one of the immediate targets of X-rays. The presented study of membrane proteins in erythrocytes of persons carrying the A-T gene was planned on the assumption that in A-T changes may occur in the membranes which would resemble those observed by electron spin resonance (ESR) in erythrocytes exposed to ionizing radiation [7] or radiosensitizing agents [8]. We have applied the same methods of protein separation, spin labelling and monitoring of ESR spectra in preparations of erythrocyte membranes to samples of blood from A-T patients and from their parents.

The presented results indicate, for the first time, that in A-T patients and heterozygotes changes may be detected in erythrocytes which differentiate them from healthy control persons. These changes in erythrocyte membranes may be caused by the same factors, which in dividing cells cause chromosomal instability and sensitivity to DNA damage.

Correspondence: A.L. Pawlak, Institute of Human Genetics, Polish Academy of Sciences, ul. Strzeszynska 32, 60-479 Poznan, Poland.

Materials and Methods

Blood from four patients with A-T (samples taken principally to study lymphocytes), ten obligatory heterozygotes of A-T (parents of patients with A-T), eight healthy children and eight healthy adult persons was collected into heparinized tubes. Blood was stored for 10 h at 4° C. After centrifugation at 1000 × *g* for 10 min at 4° C, plasma and buffy coat were removed and cells washed three times in 150 mmol/l NaCl, 5 mmol/l sodium phosphate buffer (pH 8.0). Hemoglobin was removed by osmotic lysis in 5 mmol/l sodium phosphate buffer (pH 8.0) and centrifugation at 25000 × *g* for 10 min, followed by washing five times with the same buffer. Protein was determined by the method of Lowry et al. [9].

For PAGE-SDS electrophoresis red blood cell membranes were stored frozen at −20° C. Membranes were solubilized in 125 mmol/l Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate (SDS), 20% glycerol, 10% 2-mercaptoethanol by incubation for 90 s at 100° C. 20 µl of membrane lysate containing 30 µg of protein was applied on the gel. Separation of membrane proteins was performed in 10% polyacrylamide gel containing 0.1% SDS with 4% acrylamide stacking gel [10].

Conditions of spin labelling have been chosen to minimize variation in proportion between weakly and strongly bound label [11]. The membranes in 5 mmol/l phosphate buffer (pH 8.0) were labelled with 2,2,6,6-tetramethylpiperidine-1-oxyl-4-maleimide (MAL-6) added in amount of 1 mg per 25 mg of membrane protein [12]. The mixture was incubated overnight (14 h) in dark at 4° C. To remove unreacted spin label, membranes were washed five times with 5 mmol/l sodium phosphate buffer (pH 8.0). Within 2 h after preparation the samples were transferred to the ESR laboratory and placed in 1.5 mm inner diameter quartz tubes. The ESR spectra were recorded at room temperature on RADIOPAN SE/X-2543 X-band spectrometer with TE_{102} rectangular cavity and 100 kHz field modulation. Appropriate settings of spectrometer were taken to avoid saturation effects (microwave power 16 mW), overmodulation broadening (modulation field 0.3 G), and transient spectrum deformations (time constant 0.3 s, scan time 8 min for 10 mT scan range). Spectrometer was operated at 9.4 GHz (X-band) with the field range 328.5–338.5 mT. Amplitudes of signals from weakly ('w') and strongly ('s') bound spin label were quantified as described by Barber et al. [11]. The preparation procedure, timing of experiments, and ESR spectrometer settings were kept constant for all samples.

A number of spin labels in the samples containing the specified quantity of proteins was controlled by measurements of an integral intensity of the ESR spectra and was practically constant in our samples. The *w/s* parameters can be used as a measure of a relative amount of weakly and strongly bound MAL-6 molecules in the membrane. A change in *w/s* indicates changed distribution of spin labels between two types of SH groups displaying low and high mobility. The *w/s* parameters can be used as a measure of relative amounts of weakly and strongly bonded MAL-6.

Results

Additional fraction of low electrophoretic mobility, migrating slower than spectrin on SDS-PAGE, was found in erythrocyte membranes of both A-T patient and A-T heterozygote (Fig. 1).

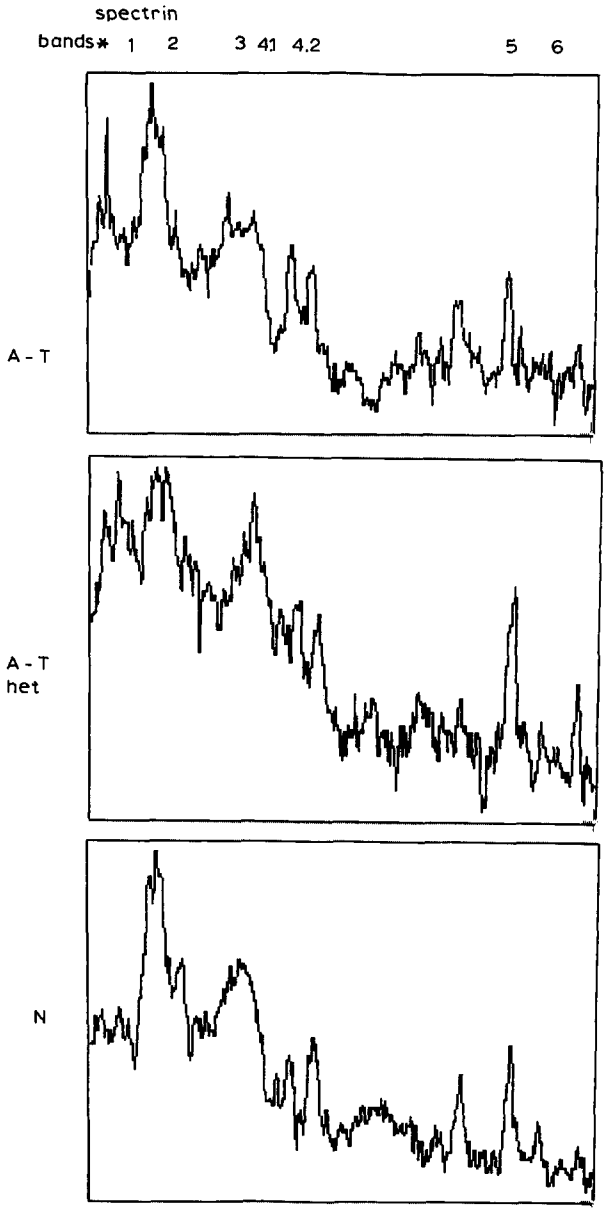

Fig. 1. SDS-PAGE densitometric scannings (Ultrascan LKB) of erythrocyte membrane proteins from ataxia telangiectasia patient (A-T), ataxia telangiectasia heterozygote (A-T het) and from normal person (N). Additional fraction present in carriers of A-T gene marked (*).

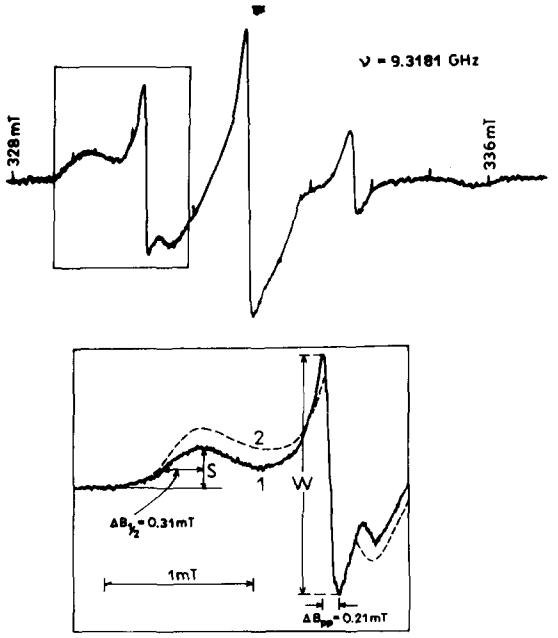

Fig. 2. ESR spectrum of MAL-6 attached to the erythrocyte membrane membrane (upper) and exemplary low-field components ($M_I = +1$) of spectra of the adult control (spectrum 1) and A-T heterozygote (spectrum 2) preparations. The $B_{1/2}$ and B_{pp} are independent of the w/s ratio.

ESR spectrum of MAL-6-labelled erythrocyte membrane of control adult person is shown in Fig. 2. It is a typical spectrum of maleimide label covalently bonded to erythrocyte membranes. This kind of spectrum is commonly recognized as a two-component spectrum [13]. The narrow line triplet is a spectrum from weakly immobilized labels with correlation time of a motion of the order of 10^{-10} s. The low-field line amplitude ($M_I = +1$) of this triplet is marked as 'w' in Fig. 2. The broad line triplet is a powder-like spectrum from strongly immobilized labels (with correlation time longer than 10^{-7} s) with low-field line amplitude marked as 's' (Fig. 2). Preparations of erythrocyte membranes were characterized by the w/s ratio. The mean value of w/s parameter in the adult control group was 6.22 ± 0.27, whereas in the group of children age-matched with A-T homozygotes it was 3.74 ± 0.33. In heterozygotes of A-T significantly lower w/s values were found as compared to the respective age-matched control group (4.15 ± 0.75, $P \leq 0.01$; Table I), whereas in homozygotes values of w/s were higher than in the control group of children (5.02 ± 0.53, $P \leq 0.05$).

The age-dependent changes in the environment of protein SH groups in erythrocyte membranes were demonstrated by an increase in the value of w/s parameter from 3.74 (± 0.75) in the group of healthy children to 6.22 (± 0.27) in the group of healty adult persons – the difference was significantly at $P \leq 0.001$. No difference in w/s values was noted when groups of A-T children ($w/s = 5.02 \pm 0.53$) and their parents, heterozygotes of A-T ($w/s = 4.15 \pm 0.75$) were compared ($P \geq 0.05$).

The ESR linewidth values B_{pp} and $B_{1/2}$ marked in Fig. 2 are not affected by the A-T gene. It indicates a lack of changes in the motional correlation time and in a distribution of the correlation times.

TABLE I

Parameters derived from ESR spectrum of erythrocyte membrane proteins labelled with MSL and characterizing relative amplitudes of weakly ('w') and strongly ('s') bound spin label

Group (number of persons)	w/s			Mean age (y)
	mean value (+S.D.)	range of values	difference vs. respective control *	
Children				
Homozygotes of A-T (4)	5.02 (±0.53)	4.4–5.6	$P \leq 0.01$	8.25 (±3.0)
Control (8)	3.74 (±0.33)	3.2–4.1		7.4 (±2.4)
Adults				
Heterozygotes of A-T (10)	4.15 (±0.75)	3.3–5.6	$P \leq 0.005$	32.3 (±3.5)
Control (8)	6.22 (±0.27)	6.0–6.8		31.1 (±2.9)

* Student's t-test.

Discussion

The presence of additional fraction of protein in erythrocyte membranes of carriers of A-T gene (Fig. 1) may explain lower values of w/s in membranes of A-T heterozygotes (Table I). On the other hand, the increase in w/s values found in A-T homozygotes may be explained by changes induced in these children by the disease process.

One can consider involvement of spectrin in occurrence of the observed additional protein fraction. Blood storage may modify spectrin in erythrocyte membranes as shown by decreased binding of actin to such preparations [12]. Spectrin, normally present in the tetramers form, may also occur in the form of branching structures or as part of spectrin-actin lattices [13] both in vivo [14] and in vitro [15].

Protein-specific spin label MAL-6 is covalently bonded to the SH-groups of proteins and leaves lipid bilayers unlabelled. The spin label senses the differences in environment of SH groups of membrane proteins. In the ESR spectra of membrane proteins labelled with MAL-6 two signals are identified as corresponding to the mobile (weakly bound = 'w' component) and immobile (strongly bound = 's' component) residues of spin label. 'w'-type sites are situated on the membrane surface in a highly polar environment, whereas the 's'-type sites are located at the membrane surface as embedded deeply in lipid bilayers [16,17]. Under standard labelling conditions MAL-6 has greater affinity for high molecular weight membrane proteins and most of

its binding sites (70–90%) is located on the skeletal proteins, spectrin and band 3 protein [17]. Changes in aggregation of spectrin, that is reversible transitions between tetrameric and dimeric forms occur parallel with changes in *w/s* value [18]. Therefore, *w/s* may serve as a marker of the physical state of erythrocyte membranes. Mobility of sulphhydryl groups of membrane proteins may be affected by changes in conformation of spectrin produced by such compound as spermine or 2,3-bisphosphoglycerate [18]. Values of *w/s* may be either decreased or increased, respectively, as result of in vitro treatment of membranes with quinolinic or glutamic acids [19].

Despite of the use of preparation procedures identical in many respects to those reported by the authors of the method [11,16], after standard 18 h incubation of membranes with MAL-6 in 5 mmol phosphate buffer followed by washing we have obtained a control value of *w/s* of 6.22, that is higher than 5.4 found by Barber et al. [11], but falls within the range of values reported by Butterfield and coauthors within the time period of 10 years (from 3.65 ± 0.12 [20] to 5.47 ± 0.49] [21]). Comings reported even higher control values of *w/s* (7.35 ± 1.95) [16]. The relatively high values of *w/s* in our study probably are not due to the experimental conditions of the determination of ESR spectra, since in this case increased *w/s* would disturb the narrow lines (*w*). On the other hand, longer duration of the stages of blood storage (10 h) and of transporting of labelled erythrocyte membranes (2 h) in our experimental set up could be the cause of increased values of *w/s* in control blood. Our results should, however, well characterize intergroup differences in membrane characteristics, since all blood samples were processed by the same procedure.

In human erythrocyte membranes the fraction of weakly immobilized residues of MAL-6 ('w') increased following exposure to denaturing agents [22], ionizing radiation [7] and heat treatment [23]. Properties of MAL-6-labelled membrane proteins were studied also in some genetic disease characterized by changes in membrane functions. Increased fractions of weakly immobilized spin label were found in Duchenne's muscular dystrophy [24–26] and in myotonic dystrophy [24]. Observation of the shortened life span of erythrocytes in patients with myotonic dystrophy [27] suggests that the described changes in erythrocyte membranes may be accompanied by disturbances of cell function. If life span of erythrocytes was shortened also in A-T patients, this would explain the increase in *w/s* found in this group (Table I).

The main point in our study is demonstration that erythrocyte membranes are changed in heterozygotes of A-T as compared to control persons.

It seems that the in vitro procedures in our study were controlled enough to exclude the possibility that some in vitro membrane-modifying factors could decrease the *w/s* value in carriers of A-T gene as it was noted with spermine [28] or quinolinic acid [19].

It is interesting to note, that the decrease in the weakly immobilized fraction of spin label ('w') seen by us in erythrocyte membranes of heterozygotes carrying A-T gene (Table I) resembles the effect of ionizing radiation on erythrocyte membranes [7] or changes produced by treatment with chemical radiosensitizers [8]. These changes may be not directly related to the presumed increased radiosensitivity of A-T heterozygotes, but may be explained in terms of recently described abnormalities in the levels of SH groups in A-T cells [29,30].

We have shown also that esr spectra of erythrocyte membranes change with age of the blood donor, the values of *w/s* being significantly higher in adult (6.22 ± 0.27) as compared to those in children's group (3.74 ± 0.33, $P \leq 0.001$). This points to the importance of matching for age which has been demonstrated by showing that the apparent differences in *w/s* between erythrocytes of patients with Huntington's chorea and normal persons [31] have not been confirmed in the study on age-matched groups [11]. On the other hand, the higher values of *w/s* seen in muscular dystrophy can not be explained by age differences, since the younger group would be expected to have lower *w/s* ratios.

By finding of additional protein fraction we have shown for the first time, that A-T patients and A-T heterozygotes can be differentiated from the healthy control persons on the basis of changes detected in erythrocytes. This offers an unique opportunity to diagnose A-T genotype on the basis of investigation of these readily accessible cells.

Diagnosis of heterozygotes of A-T is important not only for purpose of genetic counselling, but also for prevention of genotoxic exposures of these hypersensitive subjects. Tests based on in vitro challenge give variable results in heterozygotes from different families (complementation groups) [32,33]. Sensitivity to in vitro treatment with caffeine has been shown in A-T heterozygotes from four families [34,35]. Heterozygotes of A-T seem also to be significantly more sensitive to induction of micronuclei in vivo as result of natural exposure to genotoxic agents [36]. Cell lines heterozygous for A-T are consistently more sensitive to effects of ionizing radiation in G_2 [37]. When the effects of the A-T gene in heterozygotes and in homozygotes are compared, the linear relation between gene-dose and the effect are rarely observed [38].

References

1 Gatti, R.A., Berkel, I., Boder, E., Braedt, G., Charmley, P., Concannon, P., Ersoy, F., Foroud, T., Jaspers, N.G.J., Lange, K.,

Lathrop, G.M., Leppert, M., Nakamura, Y., O'Connell, P., Paterson, M., Salser, W., Sanal, O., Silver, J., Sparkes, R.S., Susi, E., Weeks, D.E., Wei, S., White, R. and Yoder, F. (1988) Nature 336, 577–580.
2 Bridges, B.A. and Harnden D.G. (eds.) (1982) Ataxia-telangiectasia. A cellular and molecular link between cancer, neuropathology, and immune deficiency, Wiley, Chichester.
3 Jaspers, N.G.J. and Bootsma, D. (1982) Proc. Natl. Acad. Sci. USA 79, 2641–2644.
4 Swift, M., Morrell, D., Cromartie, E., Chamberlin, A.R., Skolnick, M.H. and Bishop, D.T. (1986) Am. J. Hum. Genet. 39, 573–583.
5 Swift, M., Reitnauer, P., Morrell, D. and Chase, C. (1987) New Engl. J. Med. 316, 1289–1294.
6 Swift, M. and Chase, Ch. (1983) Lancet, 1049–1050.
7 Grzelinska, E., Bartosz, G., Gwozdzinski, K. and Leyko, W. (1979) Int. J. Radiat. Biol. 36, 325–331.
8 Rybczynska, M. Szczepaniak, L.S. (1986) Stud. Biophys. 116, 133–134.
9 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
10 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2506–2517.
11 Barber, M.J., Rosen, G.M. and Rauckman, E.J. (1983) Biochim. Biophys. Acta 732, 126–132.
12 Wolfe, L., Byrne, A. and Lox, S. (1986) J. Clin. Invest. 78, 1681–1686.
13 Bennett, V. (1989) Biochim. Biophys. Acta 988, 107–121.
14 Liu, S.C., Windisch, P. and Palek J. (1984) Cell 37, 587–594.
15 Morrow, J.S. and Marchesi V.T. (1981) J. Cell Biol. 88, 463–468.
16 Comings, D.E., Pekkala, A., Schuh, J.R., Kuo, P.C. and Chan, S.I. (1981) Am. J. Human Genet. 33, 166–174.
17 Butterfield, D.A., Roses, A.D., Appel, S.H. and Chesnut, D.B. (1976) Arch. Biochem. Biophys. 177, 226–234.
18 Farmer, II, B.T. Harmon, T.M. and Butterfield, D.A. (1985) Biochim. Biophys. Acta 821, 420–430.
19 Farmer, B.T. II, Nicholas, M.N. and Butterfield, D.A. (1984) Biochim. Biophys. Acta 778, 260–268.
20 Butterfield, D.A. (1977) Biochim. Biophys. Acta 470, 1–7.
21 Butterfield D.A., Wyse, J.W., Abbott, V.L. and Nonneman, A.J. (1986) Biochem. Arch. 2, 245–252.
22 Kirkpatrick, F.H. and Sandberg, H.F. (1973) Biochim. Biophys. Acta, 298, 209–218.
23 Grzelinska, E., Bartosz, G. and Leyko, W. (1982) Int. J. Radiat. Biol., 42, 45–55.
24 Butterfield, D.A. (1977) Biochim. Biophys. Acta 470, 1–7.
25 Dellantonio, R., Angeleri, F., Capriotti, M., Lenaz, G., Curatola, G., Mazzanti, L. and Bertoli, E. (1980) Ital. J. Biochem. 29, 121–128.
26 Wallach, D.F.H., Verma, S.P. and Singer, W.E. (1983) J. Exp. Med. 157, 2017–2028.
27 Sydow, O., Stibler, H. and Hast, R. (1985) Acta Neurol. Scand. 72, 522–524.
28 Wyse, J.W. and Butterfield, D.A. (1988) Biochim. Biophys. Acta 941, 141–149.
29 Meredith, M.J. and Dodson, M.L. (1987) Cancer Res. 47, 4576–4581.
30 Dean, S.W., Sykes, H.R. and Cole, J. (1988) Cancer Res. 48, 5374–5376.
31 Butterfield, D.A., Oeswein, J.Q. and Markesberry, W.R. (1977) Nature 267, 453.
32 Shiloh, Y., Tabor, E. and Becker, Y. (1982) Cancer Res. 42, 2247–2249.
33 Shiloh, Y., Tabor, E. and Becker, Y. (1982) Carcinogenesis 3, 815–820.
34 Pawlak, A.L., Kotecki, M., Ignatowicz, R. and Mruk, M. (1985) Mut. Res. 147, 312–313.
35 Pawlak, A.L., Kotecki, M. and Ignatowicz, R. (1990) Mut. Res., in press.
36 Rosin, M.P. and Ochs, H.D. (1986) Human Genet. 74, 335–340.
37 Parshad, R., Sanford, K.K., Jones, G.M. and Tarone R.E. (1983) Cancer Genet. Cytogenet. 14, 163–168.
38 Pawlak, A.L. and Ignatowicz, T. (1990) in 5th International Conference on Environmental Mutagens (Mendelsohn, M.L., ed.), Alan R. Liss, New York, in press.