CROSS-LINKING OF RED BLOOD CELL MEMBRANE PROTEINS INDUCED BY OXIDATIVE STRESS IN β THALASSEMIA

I. KAHANE

Biomembrane Research Laboratory, The Hebrew University- Hadassah Medical School, PO Box 1172

and

A. SHIFTER and E. A. RACHMILEWITZ

The Hematology Service, Hadassah University Hospital, Mount Scopus, PO Box 24032 and *the Hebrew University-Hadassah Medical School, Jerusalem, Israel*

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1. Introduction

The structure–function relationship of the normal red blood cell (RBC) membrane has been extensively studied [1] and in many ways the RBC membrane served as a model for the understanding of membrane physiology in general. Recently there is a growing interest in characterizing RBC membrane defects in several congenital hemoltyic disorders where changes in the membranes are not considered to be the primary defect of the disease, but nevertheless contribute to the pathophysiology of the hemolytic process. Alterations in membrane structure were reported in hemoglobinopathies such as sickle cell anemia [2] and in thalassemia [3,4]. In the latter syndrome, direct and indirect evidence indicate that the pathological RBC are exposed to higher oxidative stress when compared to normal RBC [5], as a result of increased production of 'activated oxygen', such as superoxide, peroxide, singlet oxygen and hydroxyl radicals [5]. These radicals eventually oxidize various RBC components including membrane proteins and lipids, as indicated by the significantly lower titratable SH groups (about 50% normal) [3], while lipid oxidation was suggested by the lower ratio of unsaturated to saturated fatty acids of membrane lipids [4]. In addition, malonildialdehyde (MDA), a secondary breakdown product of lipid peroxidation, was generated in excess amounts in thalassemic RBC, following exogenous oxidative stress with peroxide [6]. MDA is a bifunctional reagent [7] and was reported to crosslink several cell constituents, including membrane components [8]. A crosslinked membrane is expected to be more rigid, and in fact, this could be an explanation for the increased resistance to osmotic lysis of thalassemic RBC when compared to normal RBC [9]. Thus, it may be suggested that thalassemic RBC membrane components are crosslinked as a result of the increased oxidative stress that the cells are exposed to.

The present study was carried out in order to test the above hypothesis and to examine whether the effect of an exogenous oxidative stress can also result in crosslinking of thalassemic membrane proteins. In addition, the prevention of crosslinking by α-tocopherol (vitamin E) was examined, since administration of this vitamin to thalassemic patients was reported to change the osmotic fragility of the RBC to close to normal values [3]. The results indicate that:

(i) Several membrane proteins are crosslinked in native thalassemic RBC.
(ii) Under exogenous oxidative stress crosslinking of several membrane proteins is more readily seen in thalassemic RBC when compared to normal RBC.
(iii) Peripheral membrane proteins were mainly altered

by the oxidative stress while no change was found in glycophorin.

(iv) α-Tocopherol administration did not abolish the crosslinking of the membrane proteins.

2. Materials and methods

2.1. *RBC and membrane preparation*

RBC were freshly obtained from healthy donors and from patients with β thalassemia major or β thalassemia intermedia; the latter are infrequently transfused. Several patients were treated with α-tocopherol (Hoffman La Roche, Nutley, NJ) for at least 5 months with a daily dose of 750 IU/day [3]. Two healthy controls were treated with α-tocopherol (1050 IU/day for 10 days). The RBC were washed 3 times in phosphate buffered saline (PBS) and membranes were prepared as described [3].

2.2. *Oxidative stress*

Oxidative stress was applied to the RBC following the procedure used [6] to generate MDA, by addition of 8 mM H_2O_2 in PBS, to equal vol. 50% RBC suspension in PBS which includes 4 mM sodium azide. The cells were incubated for 2 h at 37°C and washed in PBS. Membranes were derived from most of the cells, while the rest were used for the estimation of MDA [6].

2.3. *Analytical procedures*

Total membrane protein was estimated as in [10], using bovine plasma albumin as a standard. Malonyldialdehyde (MDA) was estimated as described [6]. α-Tocopherol was determined as in [11]. The electrophoretic pattern of the membrane polypeptides was analysed in 5.9% acrylamide gels which contained 1% sodium dodecyl sulfate (SDS) following a modification of the procedure in [12,13]. The numenclature of the polypeptide bands is according to [12].

3. Results and discussion

3.1. *Crosslinking of membrane proteins in native thalassemic RBC*

Some quantitative differences in the intensity of the major polypeptide bands could be seen (fig.1)

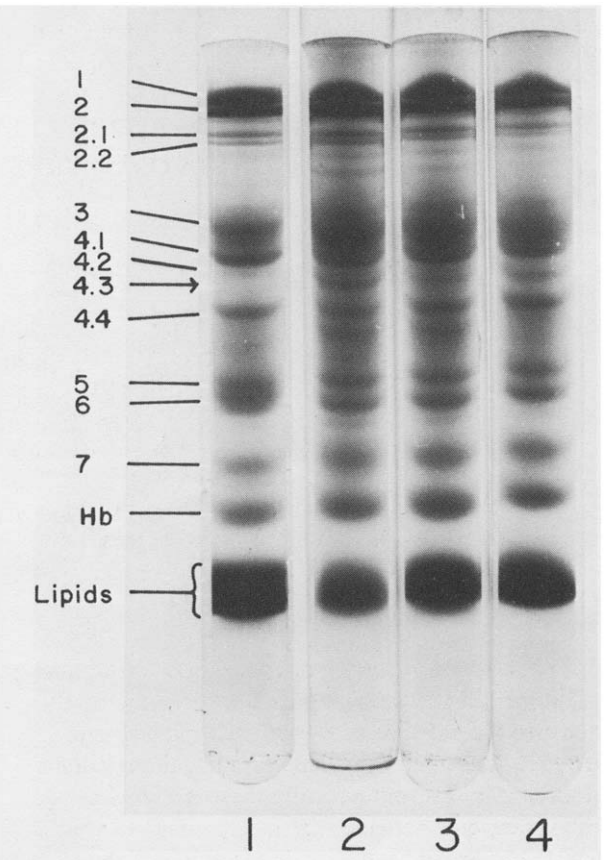

Fig.1. SDS–polyacrylamide electrophoretic profiles of membrane polypeptides from RBC of β-thalassemic patients and healthy donor. Note the differences between the profiles of 3 patients (gels 2–4) and that of a healthy donor (gel 1).

while the general electrophoretic pattern of the thalassemic RBC membrane resembled that of normal RBC membrane. However, on a more careful examination two qualitative reproducible changes were observed:

1. One extra band (band 4.3) with app. mol. wt approx. 70 000 was noticed in the electrophoretic profile of all thalassemic RBC membranes examined, while in a few control membranes only a very faint band with the same apparent molecular weight was observed. The extra polypeptide band (fig.1, band 4.3) could be a product of crosslinking of some membrane proteins. It is less likely to be cross-

linked hemoglobin, since its apparent molecular weight is different from that of dimer, trimer or a tetramer of globin chains. Although a hybrid of globin chains with membrane polypeptides cannot be excluded [14,15].

2. Aggregated polypeptides were detected only in the profile of the thalassemic RBC membranes. These aggregates were mainly on top of the gel, but were also observed along the gel as indicated by the low, but well visualized background of the protein stain between the distinct polypeptide bands (fig.1). Similar findings of aggregated RBC membrane polypeptides were reported in different experiments using a variety of crosslinking reagents at relatively low concentrations [14,16,17]. The demonstration of aggregated polypeptides in thalassemic membranes may therefore suggest the presence of crosslinking factor(s) within these cells, as a possible result of the intracellular oxidative stress.

3.2. *Crosslinking of membrane protein following exogenous oxidative stress*

Normal and thalassemic RBC were exposed to relatively low concentrations of hydrogen peroxide (4 mM). The electrophoretic pattern of the RBC membrane polypeptides derived from RBC incubated with and without H_2O_2 are demonstrated in fig.2. Major differences were observed in the electrophoretic profiles of treated thalassemic (fig.2, gel 4) when compared to normal RBC membranes (fig.2, gel 2). In the former, several bands like those of spectrin (bands 1 and 2) and bands 2.1 and 2.2 almost disappeared and the intensity of staining of band 3 markedly decreased with a concomitant increase of aggregated material on top of the gel as a result of crosslinking. The effects of H_2O_2 on similar components of normal RBC membranes were much less pronounced. In both electrophoretic profiles a marked increase was observed in the intensity of staining in the regions of bands 5 and 7. There were no differences between the thalassemic and the control RBC sialoglycoprotein (glycophorin) bands, confirming that the peptide regions of the sialoglycoprotein, which are exposed either to the cytoplasm or to the external surface of the cell [18] are not susceptible to crosslinking, in contrast to other membrane polypeptides which crosslink readily with a variety of crosslinking reagents including gluteraldehyde [14,16,17].

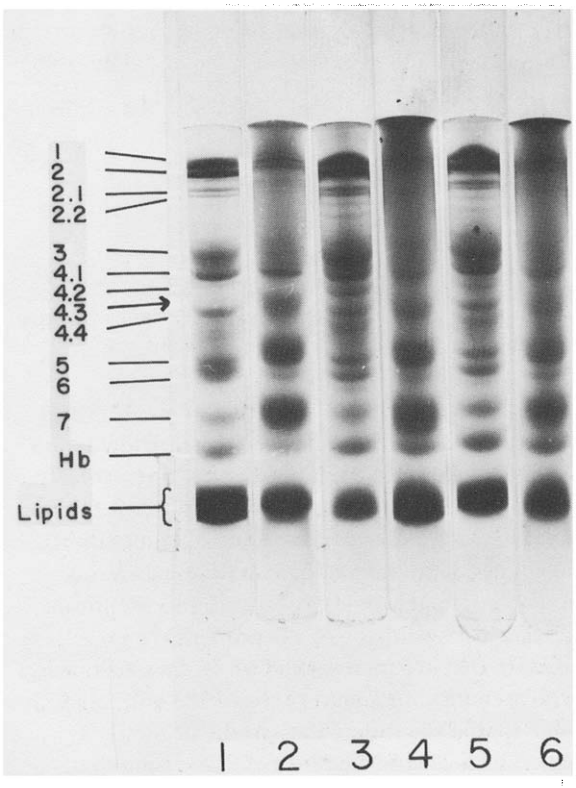

Fig.2. Crosslinking of membrane proteins following exogenous oxidative stress. Electrophoretic profiles of membranes of untreated (gels 1, 3, 5) and treated (gels 2, 4, 6) RBC are in pairs. Healthy donor (gels 1, 2); β-thalassemic patient (gels 3, 4) β-thalassemic patient who was treated with vitamin E (gels 5, 6).

One could assume that the membrane proteins were crosslinked via disulphide bridges, since low values of titratable SH groups were found in native thalassemic RBC membranes [3]. However, solubilization of the membranes in SDS in the presence of reducing agent (β-mercaptoethanol, 75 mM) did not eliminate the finding of aggregated polypeptides. Another possible crosslinking agent which may result from the intracellular oxidative stress is MDA. Under the same experimental conditions used in the present study, higher MDA levels are generated from thalassemic RBC as compared to normal RBC [6]. A decrease in MDA level to almost normal values was

Table 1
MDA generation following peroxide stress in relation to serum α-tocopherol levels in thalassemic and control RBC

RBC	α-Tocopherol administration	Serum α-tocopherol (mg %)	MDA generated (nmol/g Hb)	Corresponding gel in fig.2
Control	–	0.7[a]	145[a]	2
β-Thalassemia	–	0.4	590	4
β-Thalassemia	+	1.4	153	6

[a] Normal ranges 0.6–1 mg% and 100–200 nmol/g Hb for serum α-tocopherol and MDA generation, respectively

found following the administration of α-tocopherol to thalassemic patients, where initial very low serum α-tocopherol levels have been reported [4]. Therefore, if the increased generated MDA levels is a major factor in the crosslinking of membrane proteins of thalassemic RBC, then in patients who receive α-tocopherol one would expect to find a significant decrease in crosslinking of the proteins. The results indicated that in patients with high serum α-tocopherol levels although MDA levels were within normal range (table 1), the electrophoretic profile of the H_2O_2 treated RBC membranes did not differ from that of RBC from patients with low serum α-tocopherol levels (fig.2, gels 4 and 6).

The fact that α-tocopherol did not prevent the crosslinking of the membrane protein may support the current concept that this presumable antioxidant is located in close contact with the fatty acids of the lipid backbone of the membrane [19]. It is not yet known whether this vitamin could protect lipids from crosslinking to other lipids or proteins.

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