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PHOTODYNAMIC EFFECTS OF PROTOPORPHYRIN ON THE ARCHITECTURE OF ERYTHROCYTE MEMBRANES IN PROTOPORPHYRIA AND IN NORMAL RED BLOOD CELLS

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Summary

Protoporphyrin causes a photodynamic damage of the red blood cell membrane. After illumination of red blood cells in the presence of protoporphyrin three effects can be observed:

1. Red blood cell membranes show particle aggregation on the outer and inner fracture face, as seen in freeze-etch electron microscopy.

2. Electrophorograms of membrane proteins show an increasing protein association, not disrupted by sodium dodecyl sulfate.

3. The immunological response of A+ red cells to anti A serum is progressively retarded.

It seems likely that these effects are interrelated consequences of the protoporphyrin-induced photodynamic membrane damage.

Introduction

Hypersensitivity of the skin to sunlight is the main characteristic of patients suffering from erythropoietic protoporphyria (E.P.P.) [1,2]. It has been shown that red cells of these patients are liable to photohemolysis when exposed to light [3,4]. During illumination a progressive efflux of potassium ions, compensated by an equal influx of sodium ions is observed in the prelytic phase, followed by hemolysis. From experiments with normal intact red blood cells it was concluded that the lysis is induced by protoporphyrin, a compound which is present in high concentrations in the red cells of the patients [5].

The damaging effect on the erythrocyte membrane appeared to be linked to the oxidation of amino acid residues in membrane proteins rather than to the oxidation of unsaturated membrane fatty acids [6,7]. Since proteins are related to intra-membrane particles which are visible on fracture faces of

freeze-etched membranes, it could be argued that these substructures are possibly affected by the exposure of the membranes to protoporphyrin and light, thus offering the opportunity to visualize defects in the membrane ultrastructure.

In this paper it will be shown that photooxidation of red cells in the presence of protoporphyrin leads to a rearrangement of intra-membrane particles and ultimately to a severe mutilation of the membrane, with a concomitant change of the electrophoretic behavior of isolated membrane proteins.

Methods

Heparinized blood was centrifuged, washed and resuspended as described previously [7]. Ghosts were prepared according to the method described by Weed et al. [8]. Illumination was carried out at pH 7.4 with a 125 W super high pressure mercury lamp (Philips, type HPL), placed at a distance of 10 cm from a lens. A parallel beam of light was reflected through the bottom of the incubation vessel by a mirror. E.P.P. red blood cell suspensions (10% cells) were illuminated as such, whereas ghost suspensions at a final concentration of 80% were supplemented with protoporphyrin (100 μg/ml), as protoporphyrin is completely washed out during ghost preparation. The observations on intact cells were limited to the first part of the prelytic phase, in which 75% K^+ was lost. Beyond this point hemolysis starts rapidly [5].

Samples for freeze-etching were prepared as described before [9]. Specimens were freeze-fractured and etched in a Denton machine. The replicas were stripped off on distilled water, cleaned with a hypochlorite solution (2% active chlorine) and examined in a Philips E.M. 200.

Sodium dodecyl sulphate electrophoresis of membrane proteins was performed with the method described by Fairbanks et al. [10], after solubilization of the stroma in 10 mM Tris/HCl, containing 1% sodium dodecyl sulphate (SDS), 40 mM dithiothreitol and 1 mM EDTA.

The immunological response of intact cells after illumination was studied on A+ red blood cells. Illuminated cells were mixed with anti A serum, the concentration of which was adjusted to give microscopically visible agglutination 10—15 seconds after mixing with non-illuminated cells.

Results and Discussion

Membranes of intact erythrocytes and ghosts display the well known freeze-etch image of fracture faces covered with randomly distributed particles (Fig. 1, A and B). Aggregation of particles was observed when normal red cell membranes were illuminated during 15 minutes in the presence of protoporphyrin (Fig. 1, C and D). A similar membrane ultrastructure was visible during the second half of the prelytic phase of illuminated intact cells from patients. Membranes exposed to light for 30 minutes displayed a strongly mutilated ultrastructure, which did not permit a detailed analysis in terms of number of particles and area of particle-free fracture regions (Fig. 2).

Membranes illuminated in the absence of protoporphyrin and membranes incubated with protoporphyrin in the dark displayed a normal freeze-etch image.

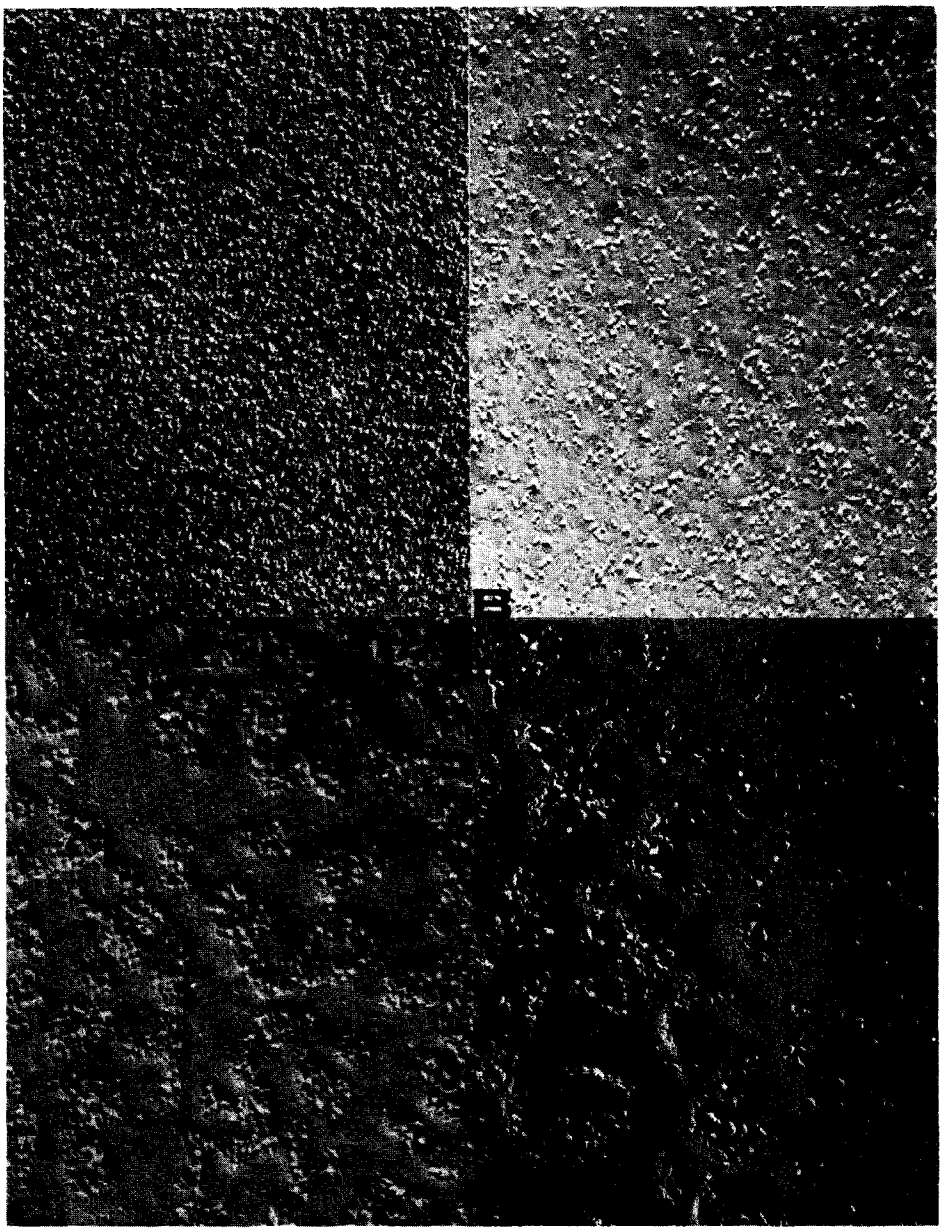

Fig. 1. A and B. Fracture faces of erythrocyte ghosts typical for material illuminated in the absence of protoporphyrin or kept in the dark in the presence of protoporphyrin. A, inner fracture face; B, outer fracture face. C and D. Fracture faces of erythrocyte ghosts illuminated during 15 minutes in the presence of protoporphyrin. C, inner fracture face, D, outer fracture face. Magnification: about 80 000×.

Concomitantly it was found that the spectrum of erythrocyte proteins was altered considerably by illumination in the presence of protoporphyrin. Sodium dodecyl sulphate electrophoresis of non-illuminated membrane proteins yielded the normal pattern (Fig. 3, A). With increasing illumination

Fig. 2. Fracture face of erythrocyte ghost illuminated during 30 minutes in the presence of protoporphyrin. Magnification: about 80 000×.

periods it appeared that the protein bands became blurred, whereas increasing amounts of protein remained on top of the gels (Fig. 3, B—E). This indicates a progressive association of membrane proteins during illumination. It is known that some non-covalent bonds in proteins are not dissociated by SDS [11]. Therefore it could not be decided whether this association is caused by covalent cross-linkage or by non-covalent intermolecular bonds.

Aggregation of particles in erythrocyte membranes can be provoked by incubating the cells at pH 5.4, or by treating them with proteolytic enzymes like pronase and trypsine. From these and immunolabeling experiments it was concluded that intra-membrane particles are related to lipid-protein associations [12] and most likely to glycoproteins [13]. Recently this idea was strongly supported by reconstitution experiments in which a part of the erythrocyte MN glycoprotein was incorporated in lipid bilayers [14].

From our freeze-etch experiments alone, which show aggregation of particles, it can not be concluded that the protein part of the membrane is modified. Also changes in the lipid environment of the proteins might induce the aggregation of intramembranous particles [15,16]. Considering the biochemical experiments indicating that oxidation of the fatty acids is negligible [7] and the changed electrophoretic behavior of proteins as described above, we suggest that the primary cause for the change in the membrane architecture is the modification of the protein moiety.

It has been suggested that the reversible aggregation of intramembranous particles at pH 5.4 represents an intramembrane precipitation at the isoelectric point of membrane proteins [17]. According to this view the primary effect of photooxidation might be an upward shift of the isoelectric point of the

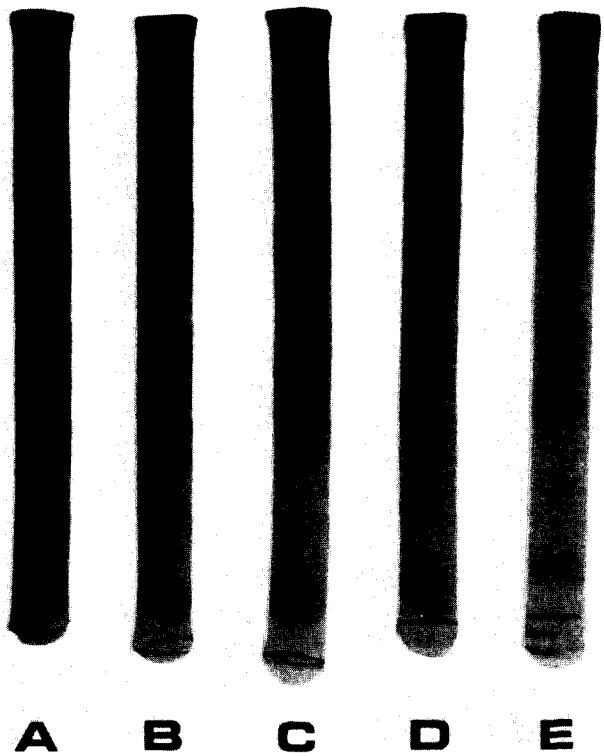

Fig. 3. Sodium dodecyl sulphate electrophoresis of erythrocyte membrane proteins, illuminated in the presence of protoporphyrin during: A, 0 minutes; B, 5 minutes; C, 15 minutes; D, 30 minutes; E, 45 minutes. Ghosts illuminated in the absence of protoporphyrin and ghosts incubated with protoporphyrin in the dark revealed a normal band pattern (A).

oxidized membrane proteins. To investigate this possibility, ghost suspensions were illuminated in the presence of protoporphyrin at pH 7.4 and then adjusted to pH 9.0. Subsequently freeze-etching and protein electrophoresis were carried out at this pH. Both freeze-etching and protein electrophoresis revealed the same pattern as at pH 7.4. Moreover, protein electrophoresis carried out at pH 5.4 yielded a normal band pattern. Thus, the observed modified protein pattern shown in Fig. 3 cannot be attributed to a process similar to that underlying the ultrastructure modification observed by Pinto da Silva at pH 5.4 [17].

Considering the relationship between antigenic properties and the freeze-etch ultrastructure of the membrane [13], we investigated whether the immunological properties of intact E.P.P. cells were affected by illumination. It appeared that in the second half of the prelytic phase the immunological response was progressively retarded. The interval between mixing of A+ E.P.P. cells with anti A serum and microscopically visible cell-agglutination increased gradually from about 10 seconds in non-illuminated cells to about 2 minutes. The strongest retardation of the immunological response was observed shortly before the onset of hemolysis, when about 75% of the intracellular K^+ had leaked out.

With normal red blood cells, sensitized by protoporphyrin added to the medium, similar results were obtained both in freeze-etch, electrophoretic and immunological studies.

In conclusion these experiments reveal that as a consequence of protoporphyrin-induced photooxidation of membrane proteins three phenomena can be observed: aggregation of particles on outer and inner fracture faces of freeze-etched membranes, protein association as seen on electrophorograms and a retarded immunological response. It seems likely that these phenomena are interconnected, although the exact mechanism is still obscure.

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