The Density and Distribution of Intramembrane Particles in Erythrocytes from Persons with Muscular Dystrophies

Joann Bodurtha, Tadayasu Hiraoka, Kichiko Koike, Masahiko Koike, Kazutake Mori, Ryuji Hazama, Tooru Hoshino and Mitsuko Nagasawa

Department of Pathological Biochemistry, Atomic Disease Institute, Nagasaki University School of Medicine, Nagasaki 852, National Kawatana Hospital, Kawatana, Nagasaki-ken 859-36 and Electron Optics Division, JEOL Ltd., Akishima, Tokyo 196


Intramembrane particles in the freeze-fractured faces of erythrocyte membranes from persons with several different types of muscular dystrophy are examined. The density of particles in the protoplasmic face was lower than that of controls, although not at a statistically significant level. No altered pattern of aggregation of these particles was observed.

A great deal of recent work in membrane biology has focused on the structural arrangement of proteins and lipids within the membrane. The freeze etching technique has been shown to split membranes during the fracture process so that two new fracture faces from the hydrophobic interior of the membrane can be seen (Pinto daSilva and Branton 1970). In most membranes the planar continuity of the fracture face has been observed to be interrupted by numerous intramembrane particles whose number, size, and array vary with membrane type and preparatory technique. These approximately 80-100 Å particles most likely represent proteins or protein aggregates with phospholipid intercalated in the membrane lipid bilayer (Branton and Deamer 1970), probably the principal integral proteins (glycophorin and band III component) interacting with spectrin at the membrane inner surface (Pinto daSilva and Nicolson 1974; Yu and Branton 1976). These particles have been shown to have translational mobility in RBC ghosts with reversible aggregation in media with an acidic pH (Pinto daSilva 1972). Drugs and ATP concentrations have also been shown to have an effect on intramembrane particle aggregation (Gazitt et al. 1977). Elgsaeter and Branton (1974) have shown that after spectrin depletion, particle aggregation in the
membrane plane may be induced by conditions which cause little aggregation in freshly prepared ghosts, suggesting that spectrin molecules form a molecular meshwork which limits the translational mobility of the RBC intramembrane particles. Elgsaeter et al. (1976) proposed that precipitation of residual spectrin molecules (following exposure to conditions which precipitate extracts of spectrin and actin) into small patches on the cytoplasmic surface of the ghost membrane is the cause of particle aggregation. It is important to remember that technical aspects of glutaraldehyde fixation and glycerol cryoprecipitation may affect the density of particles visualized by freeze-fracturing (Pricam et al. 1977).

Several groups of investigators have looked at the density and distribution of intramembrane particles in dystrophic muscle and in RBCs from organisms with dystrophy. Schotland et al. (1977) observed nonuniform distribution and depletion of particles on both protoplasmic and extracellular faces of the muscle plasma membrane in freeze-fracture studies of skeletal muscle from eight patients with Duchenne muscular dystrophy. Shafiq et al. (1976) found reduction of particles in both fracture faces and greater clustering of them in RBCs of chickens with hereditary muscular dystrophy. In freeze-fracture studies of erythrocytes from patients with Duchenne muscular dystrophy, Wakayama et al. (1978) described depletion of particles on both fracture faces of the plasma membrane. At the time of our experiments in Japan only Shafiq et al.'s results were available.

**METHODS**

**Sample selection**

In all of the studies heparinized blood and consent were obtained from patients with muscular dystrophy who lived at the National Kawatana Hospital, Nagasaki-ken, Japan, their mothers, and age-matched outpatient controls without neuromuscular or hematologic disease at the Nagasaki University Hospital. Diagnoses of each type of muscular dystrophy—Duchenne, facioscapulohumeral, and limb-girdle—had been made by at least three neurologists on the basis of clinical presentation and pedigree information. Each case conformed to the classification criteria of Walton (1969). Description of carrier status followed the definitions of Thompson et al. (1971). Definite carriers are defined as mothers of affected sons who have an affected brother or uncle; probable carriers have two or more affected sons with no affected brothers or uncles; and possible carriers have an affected son or an affected brother.

**Freeze-etching of erythrocytes**

Small aliquots of the RBCs prepared for scanning electron microscopy were removed prior to OsO₄ treatment, and, instead, infiltrated with 40% glycerol in 0.1 M phosphate buffer (pH 7.35) for several hours at 4°C. Each sample was mounted on a specimen block and rapidly frozen in Freon-22 (−160°C). Freeze-fracture replicas were made in a JEOL model JEE 4C apparatus with platinum-carbon. Replicas were cleaned in bleach, mounted on Tonbridge 400 mesh, and examined in a JEOL model 100B electron microscope at JEOL Ltd.

Two of four photographs of the protoplasmic (P) face (Branton et al. 1975) of erythrocytes in each sample were examined at a final magnification of ×100,000. Blinded particle counts were done on 10 random 2 × 2 cm² fields of each face in areas where the length of shadows of the particles were similar. Only particles clearly “protruding” from the membrane in areas free of freeze-etching artifacts were included.
RESULTS

Particle size in the P face of erythrocyte membranes in both patients and controls varied from about 50 to 100 Å in diameter with the majority being of about 90 Å. Particles were distributed throughout the membrane without any recognizable pattern of aggregation.

There was no statistically significant difference in the density of the intramembrane particles between dystrophy patients and controls, although counts of particles on the P faces of these patients’ erythrocytes were consistently low (Table 1). Densities in facioscapulohumeral and limb-girdle dystrophy showed overlap with controls which themselves gave wide variation between samples. Fig. 1 shows the freeze-etched faces of RBCs from a Duchenne muscular dystrophy patient and a control.

<table>
<thead>
<tr>
<th>Table 1. Density of intramembrane particles in the P face</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patients</strong></td>
</tr>
<tr>
<td>Duchenne muscular dystrophy (n=3)</td>
</tr>
<tr>
<td>Facioscapulohumeral dystrophy (n=3)</td>
</tr>
<tr>
<td>Limb-girdle dystrophy (n=3)</td>
</tr>
<tr>
<td>Distal myopathy (n=1)</td>
</tr>
<tr>
<td>Controls (n=4)</td>
</tr>
</tbody>
</table>

* Standard deviations expressed as per cents.

DISCUSSION

In contrast to the findings of Wakayama et al. (1978), no significant difference in density or increase in aggregation was found in the freeze-etch replicas examined in this study. While the densities computed from each photograph of a single sample varied by up to five per cent, the controls had the largest overall standard deviation and a mean value (approximately 3,800 particles per μm²) rather lower than that reported elsewhere (4,500 particles per μm², Tillack et al. 1972; and 4,200 particles per μm², Pinto daSilva et al. 1971). Bessis (1973), however, cites a broad range (2,600 to 3,800 particles per μm²). Wakayama et al. (1978) report a range of approximately 1,300 to 2,000 particles per μm² for both normals and Duchenne dystrophy patients. The number of these particles may vary from person to person (or RBC to RBC) over time as well as with experimental conditions. Serum factors, such as muscle enzymes, antibodies, or inorganic compounds might alter the intramembranous ultrastructure of RBCs.

Although others’ findings seem to support to a greater degree the concept that generalized membrane abnormalities are present in Duchenne dystrophy, the data presented in this paper might be consistent with the presence of a primary membrane defect in muscular dystrophy. However, none of the electron microscopic findings were specific for a particular type of dystrophy nor sufficient to distinguish a dystrophic sample from that of a control. Additional detailed
Fig. 1. Freeze-etched replicas of erythrocyte membranes from a patient with Duchenne muscular dystrophy (A and B) and a control (C and D). The arrows indicate the direction of shadowing.
investigation of the structural relationships of erythrocyte membrane constituents from normals and patients is needed.

Acknowledgment

This work was undertaken while Joann Bodurtha (J.B.) was supported by a fellowship from the Henry R. Luce Foundation, New York, U.S.A. and supported in part by a grant from the Ministry of Education, Science and Culture of Japan. Address of J.B. is Children Hospital of Philadelphia, Philadelphia, PA 19104, U.S.A.

References