Review Article

ERYTHROCYTES IN DUCHENNE MUSCULAR DYSTROPHY

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Abstract

This review describes erythrocyte as a model cell for biochemical investigations in the pathogenesis of muscular dystrophies. This account also deals with the enzymatic abnormalities in Duchenne muscular dystrophy and the abnormal effect of the cardiac glycoside ouabain on membrane ATPase. Moreover the importance of the latest findings on the cell shape, deformability, microviscosity and changes in surface properties are signified. Abnormal ion fluxes, fatty acid patterns and altered levels of membrane protein phosphorylation have also been discussed.

Introduction

Muscular dystrophies are the severe forms of muscle diseases with known etiology. These are genetically determined, progressively degenerative and are primary in nature; but the real cause of these disorders remains obscure.

The most serious and the most investigated type amongst the common muscular dystrophies is the Duchenne dystrophy. However the pathogenesis of the disease is still unknown (Walton and Gardner-Medwin, 1974; Rowland, 1976).

A considerable amount of biochemical findings has been accumulated during the last two decades (Pennington 1974). However, because of the great difficulty in obtaining suitable human biopsy material, comparably little information on biochemical changes in dystrophic muscle is available. Thus, on one hand, this encouraged investigators to find appropriate animal models and on the other hand inspired workers to use non-muscle cells, such as erythrocytes or skin fibroblasts in the investigation of the pathogenesis of muscular dystrophies. Obviously, erythrocytes for the reasons of availability and simplicity are now the focus of major biochemical investigations in research in muscular dystrophy.

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Table: Erythrocyte Abnormalities in Duchenne Muscular Dystrophy

Reported Abnormalities	*References*
Decreased glycolysis	Corsini and Cacciari (1958).
ATPase: abnormal response to ouabain	Brown et al. (1967).
Increased activities: hexokinase, aldolase, Pyruvate kinase.	Bosia et al. (1971).
Acetylcholinesterase: abnormal K_m and response to inhibitors.	Watts et al. (1972).
Fatty acids: altered patterns in membrane phospholipids.	Kunze et al. (1973).
Increased K^+ — efflux.	Howland (1974).
Abnormal shape.	Matheson and Howland (1974).
Increased K^+ — influx.	Sha'afi et al. (1975).
Decreased deformability.	Percy and Miller (1975).
Increased membrane protein phosphorylation.	Roses et al. (1975).
Increased phopholipase A_2	Iyer et al. (1976).
Increased electrophoretic mobilities.	Bosmann et al. (1976).
Increased lysophosphatidylcholine and diphosphatidylglycerol.	Kalofoutis et al. (1977).
Decreased ATP.	Solomons et al. (1977).

There were several investigators who have for the first time reported abnormalities in erythrocytes of Duchenne patients (Table) and subsequently several other workers in the field have evaluated these findings.

Erythrocyte enzymes:

The first report which suggested the possibility of the use of erythrocyte in muscular dystrophy research was from Corsini and Cacciari (1958). The basic theme of this report was to investigate the glycolytic status of Duchenne erythrocytes. Since, an earlier report (Dreyfus et al., 1956) suggested a low level of glycolytic enzymes in dystrophic muscle. Corsini and Cacciari (1958) reported a low glucose utilization rate in red cells from Duchenne patients. The aldolase activity of these erythrocytes was also low. However, Pennington and Loyburn (1960) found no significant change in glucose utilization by Duchenne erythrocytes.

Bosia et al. (1971) measured activities of some glycolytic enzymes in erythrocytes of Duchenne patients and reported significantly increased levels of hexokinase, aldolase and pyruvate kinase. The activities of phosphofructokinase, glyceraldehyde — 3 — phosphate dehydrogenase, phosphoglycerate kinase and lactate dehydrogenase were not significantly altered. The authors also assessed the effect of ouabain, a cardiac glycoside, on glycolysis in erythrocytes of Duchenne dystrophy. The glycolytic metabolites were not affected by ouabain in dystrophic red cells; while in normal erythrocytes fructose-1, 6-diphosphate and dihydroxyacetone phosphate were increased. Measurements of ATP in normal erythrocytes incubated in low and high potassium media (5 and 40 mm respectively) in the presence of ouabain showed an increased ATP content; while dystrophic erythrocyte ATP level remained unchanged in high potassium and decreased in low potassium medium.

The total adenosine triphosphatase (ATPase) activity of normal human erythrocyte membranes measured in the presence of Mg^{++}, Na^+ and K^+ is partly inhibited by a low concentration of ouabain. Since, the drug completely inhibits the Na^+, K^+, Mg^{++} — ATPase (the enzyme which constitutes the Na^+ and K^+ pumping system), whereas another ATPase which requires only Mg^{++} for its activity is not affected by ouabain. Brown et al. (1967) and Chattopadhyay and Brown (1972) reported that the ATPase activity of erythrocyte membranes from dystrophic patients was, by contrast, strongly stimulated by ouabain (10 M). The abnormality was also found in spontaneous muscular dystrophy, myotonia, limb-girdle dystrophy and was manifested by most but not all patients. Similar findings were reported by Poter et al. (1969) and Araki and Mawatari (1971). However, Klassen and Blostein (1969) tested seven cases of Duchenne dystrophy and found no such abnormality. The medium used by Brown et al. (1967) for ATPase assay contained approximately 3mM Na^+ and 2mM K^+ which were

insufficient for maximum activity of Na^+, K^+, Mg^{++} — ATPase. Similar concentrations of these ions were also used by Poter et al. (1969) and Araki and Mawatari (1971). On the other hand Klassen and Blostein (1969) used 50mM Na and 10 mM K^+, the optimal concentrations for Na^+, K^+, Mg^{++} — ATPase assay. Recently, Nielbroj-Dobosz (1976) showed an abnormal Na^+ — K^+ — Mg^{++} — ATPase activity which was stimulated by ouabain.

Siddiqui and Pennington (1977b) confirmed that erythrocyte membrane ATPase responds abnormally to ouabain when assayed in high as well as low salt media. Although, the authors were unable to observe any consistent stimulation of ATPase by ouabain. However, mean percentage inhibition of ATPase by the cardiac glycoside in Duchenne erythrocyte membranes was significantly less than in the normal erythrocyte membranes. This finding was also consistent with the observation of Mawatari et al. (1976). However, a recent report by Pearson (1978) has again showed that ATPase activity of erythrocyte membranes from Duchenne patients was stimulated by ouabain.

The possible cause for the abnormal effect of ouabain in Duchenne erythrocyte membrane preparations remains difficult to explain. The proposal (Palmer et al. 1966) that there could be two different types of ouabain binding sites, one eliciting stimulation and the other inhibition: then it may be assumed that the dystrophic erythrocyte membranes either contain more stimulatory sites and less inhibitory sites for ouabain binding than normal erythrocyte membranes; or the conformational state of dystrophic red cell membranes being so changed that more stimulatory sites are exposed while the inhibitory sites become unavailable. It may also be said that dystrophic erythrocytes are extremely susceptible to conformational changes produced during membrane preparation in vitro. Possible involvement of Ca^{++} in abnormal ouabain effect has also been suspected by Siddiqui (1978).

There is no evidence that the acetylcholinesterase activity in dystrophic erythrocyte is altered or changed. Nevertheless, Watts et al. (1972) reported a change in the behaviour of this enzyme towards its inhibitors. Kinetic studies on dystrophic and normal erythrocyte showed that nialamide, a competitive inhibitor of acetylcholinesterase, was not inhibiting this enzyme in dystrophic erythrocyte preparations. Eserine, on the other hand, inhibited the dystrophic enzyme under the conditions where the normal enzyme was activated. Later Das et al. (1976), using slab gel electrofocussing, claimed altered properties of Triton-solubilized acetylcholinesterase from Duchenne patients. Similar observation was reported by Goedde et al. (1977).

Iyer et al. (1976) reported a 60% elevated activity of phospholipase A in Duchenne erythrocytes. This enzyme is fairly specific for L — α — lecithin and catalyzes the hydrolysis of fatty acid in the 2 (β) position, producing lysolecithin. The concentration of lysolecithin in erythrocyte membrane is absolutely critical; since it is supposed to be a potent agent for the shape changes in erythrocyte.

Shape, Deformability, Microviscosity and Surface Properties of Erythrocyte:

It is generally believed that erythrocytes in circulation or in vitro may undergo a variety of shape alterations, both reversable and irreversable types. The discocyte-echinocyte transformation for example, is a well known reversable shape change (LaCelle et al., 1976). In several disease conditions, particularly, haematological disorders red cells undergo shape changes.

In muscular dystrophy, the first investigation (Morse and Howland, 1973) on erythrocyte shape changes was in the mouse, showed that a large proportion of the cells from the dystrophic animals were irregular in shape, involving variable protrusions from the cell surface. Matheson and Howland (1974) reported results of the scanning electron microscopic studies on red cells from different muscular dystrophy patients as well as from female carriers of Duchenne dystrophy. In every case a high proportion of distorted cells, echinocytes, were present. The highest proportion of distorted cells was reported for Duchenne dystrophy. The authors attributed these alterations in shape due to changes in the lipid of red cell membranes. Miale et al. (1975) and Lumb and Emery (1975) confirmed these findings to some extent.

With a slightly changed method, Howells (1976) showed that the echinocyte were significantly more numerous in patients with Duchenne dystrophy. Miller et al. (1976) tried to observe unmanipulated erythrocytes from Duchenne patients, female carriers and myotonic subjects by scanning electron microscopy. Their results were different from Metheson and

Howland (1974) in that they observed a large increase in stomatocytes rather than echinocytes. However, abnormal shape of dystrophic erythrocytes is a matter of controversy, and doubts about the reproduceability of these methods are severe (Matheson et al., 1976).

Percy and Miller (1975) reported a reduced deformability of erythrocyte membranes from Duchenne subjects as well as female carriers. They used micropipettes of internal diameter 1 — 2 u m and applied negative pressure to aspirate the erythrocyte. The Duchenne erythrocytes showed a reduced deformability and the authors were of the opinion that these changes were a consequence of a generalized membrane defect in muscular dystrophies.

It is now widely accepted that the bilayer plasma membranes are fluid and this can be measured in terms of microviscosity of membranes. Sha'afi et al. (1975) measured the microviscosities of erythrocyte membranes as well as muscle sarcolemma and liver cell membranes from dystrophic chicken and observed an elevation of microviscosities.

Another attempt was made to investigate surface properties of erythrocytes from dystrophic patients as well as from dystrophic animals. This was reported by Bosman et al. (1976) who used the technique of particle electrophoresis and showed that the electrophoretic mobilities of dystrophic erythrocytes were greatly increased. The electrophoretic mobility of red cells is indicative of average surface charges on a cell. In other words, increase or decrease in mobility could be the function of increasing or decreasing glycoconjugate sialic acid residue, a redistribution of negatively charged sites and changes in the hydrodynamic plane of shear alone or in combination. Since, Bosman et al. (1976) believed that the mobilities were not influenced or altered by changes in the cell shape characteristics. Thus a correlation of mobility changes with shape alteration of red cells in muscular dystrophies was not drawn. In contrast, Sato et al. (1975) found that electrophoretic mobility of erythrocytes was greatly affected by the induced shape changes.

Ion Fluxes in Erythrocytes:

Howland (1974) showed that Duchenne erythrocytes effluxed five times more potassium than normal cells. The author also observed that ouabain was without any influence on this efflux while K^+ — conducting antibiotic valinomycin stimulated the efflux rate and no consistent difference remained between Duchenne and control cells having valinomycine treatment. Following this method of Howland (1974) as closely as possible, Siddiqui and Pennington (1977a) confirmed the increased K^+ efflux in Duchenne erythrocytes. Since the efflux was unaffected by ouabain (Howland 1974), it can be concluded that the efflux observed was actually passive leak of K^+ from dystrophic red cells. Such abnormally high leak of K^+ from cells can be seen in conditions which deplete their ATP following treatment of cells with metabolic inhibitors like iodoacetate, or after incubation of the cells at 37°C without energy source for prolonged periods. In these situations Ca^{++} enters the cells and increases K^+ — efflux. Since, the intracellular level of K^+ seems to be unchanged, it can be assumed that a compensatory system is in operation in Duchenne erythrocytes which counterbalances high K^+ leak from the cells. Sha'afi et al. (1975) reported a 16% higher K^+ — influx rate in Duchenne erythrocytes than in normal cells. If the K^+ — influx is the reflection of pump activity, the Na^+, K^+, Mg^{++} — ATPase activity (ouabain sensitive) should be higher in Duchenne erythrocyte than normal cells. Presumably, however, the magnitude of the increase in K^+ — influx rate in Duchenne would be the same as that of K^+ — efflux, if measured under the same conditions. However a firm conclusion cannot be drawn at this stage.

Some studies of Ca^{++} — promoted K^+ — efflux of erythrocytes from Duchenne patients were reported by Appel and Roses (1977). The initial K^+ concentrations (m moles/litre packed cells) were not different in dystrophic cells from that of controls. With added iodoacetic acid and adenosine, no change in K^+ — efflux noted until Ca^{++} was introduced into the medium which increased the K^+ — efflux significantly in Duchenne erythrocytes. Siddiqui (1978) studied the behaviour of Duchenne and normal red cells in the condition where Ca^{++} entry into the cell was made possible. In this situation the K^+ — efflux rate was increased in both normal and Duchenne erythrocytes by about 180% while the initial difference in K^+ — efflux between the two groups remained significant. This contradicts the finding of Appel and Roses (1976) that K^+ — efflux was higher in Duchenne erythrocytes only when promoted by Ca^{++}.

Lipids in Erythrocyte Membranes:

Abnormalities in red cell membrane lipids were reported by Kunze et al. (1973) who showed that the fatty acid patterns of phosphatidylethanolamine and sphingomyelin were significantly altered in Duchenne erythrocyte membranes. In the phosphatadylethanolamine the dimethylacetals were increased, while arachidonic acid was decreased. In the sphingomyelin fraction the fatty acids 16:0, 24:1 and 18:2 were diminished and the percentage of stearic acid was elevated. The in vitro incorporation of linoleic acid into phosphatidylcholine and phosphatidylethanolamine fractions of dystrophic erythrocyte membranes was increased.

Kalofoutis et al. (1977) measured the major lipid classes as well as individual phospholipid in erythrocyte from Duchenne subjects. It was reported that phosphatidylcholine was significantly decreased, while lysophosphatidylcholine, spingomyelin and diphosphatidylglycerol were significantly increased. However this decrease in phosphatidylcholine and increase in lysophosphatidylcholine could be a result of the high phospholipase activity (Iyer et al., 1976) in Duchenne erythrocyte. Recently, a significant decrease in palmiloleic acid in the fatty acid pool of erythrocyte membranes from Duchenne patients as well as the female carriers was observed by Howland and Iyer (1977).

Phosphorylation of Erythrocyte Membrane Protein:

Roses et al. (1975) reported that the phosphorylation of spectrin band II by γ — P^{32} ATP was significantly higher in erythrocyte membranes from Duchenne patients compared with controls. The level of phosphorylation of the same component in myotonic dystrophy, was lower than controls. In further studies, Roses et al. (1976) reported a high endogenous phosphorylation of spectrin band II in female carriers of Duchenne muscular dystrophy. It seems that the authors intend to correlate the structural similarities between spectrin and myosin in humans. Since, the muscle fiber degenerates in muscular dystrophies, it can be speculated that myosin of muscle fiber is defective and that the same gene is perhaps involved in controlling the spectrin and possibly one form of the heavy chain of myosin. The intracellular phosphorylation of adenine nucleotides derived from adenine incorporated by the erythrocyte was also reported abnormal in Duchenne muscular dystrophy (Solomons et al., 1977).

General Comments

If we look at the general functional capacities of erythrocytes from Duchenne patients, it can be observed that these cells appear to be normal. The glucose utilisation rate was normal (Pennington and Loyburn, 1960), ATP level was unaltered (Bosia et al., 1971), the life span appears to be normal (Adornato et al., 1977) and haemoglobin efflux was unaltered (Siddiqui 1978) which suggests that Duchenne erythrocytes are perfoming many vital functions in the normal way.

Many abnormalities reported for Duchenne erythrocyte, e.g. reduced deformability (Percy and Miller, 1975) increased electrophoretic mobilities (Bosman et al., 1976), increased phosphorylation of spectrin band II (Roses et al., 1976), changes in membrane lipids (Kunze et al., 1973) are in some other conditions associated with the presence of morphological changes in erythrocytes. For Duchenne dystrophy, there is no good evidence that the circulating erythrocytes are deformed. If the statement that Duchenne erythrocytes are less deformable is correct, then it would be expected that the life span of these cells would be shorter, which does not seem to be the case.

There is evidence that phosphorylation of spectrin band is directly associated with erythrocyte shape (Sheetz and Singer, 1977) and the work of Greenquist and Sohet (1975) showed pronounced reduction in the level of phosphorylation occurred in hereditary defects of red cell shape like the hereditary spherocytosis and the hereditary stomatocytosis. If it is true that with the change in the cell shape phosphorylation decreases, then elevated phosphorylation of spectrin band II in Duchenne is difficult to explain. Perhaps it is important at this stage to correlate the degree of phosphorylation of spectrin with the type of change in the cell shape (e.g. endocytotic or exocytotic types).

A change in permeability, microviscosity and in the conformational state of cell membrane could result from the reported elevation of membrane phopholipase A_2 activity (Iyer et al., 1976). In this situation, levels of lysophosphatidylcholine would be raised and phosphatidylcholine decreased. That is what Kalofoutis et al. (1977) reported for Duchenne erythrocytes. Accumulation of lysophosphatidyl-

choline could damage the membrane. To overcome this crisis, the possibility could be either to extrude the lysophosphatidylcholine from the membrane or to reconvert it into phosphatidylcholine by acylase. It will be interesting to see whether the activity of acylase in Duchenne erythrocytes is altered. The question arises as to how phospholipase A_1 becomes elevated. It is known that Ca^{++} is required for phospholipase A activity and it may be possible that the Duchenne erythrocytes contain more Ca^{++} than normal.

In conclusion it may be said that at the present time, on the basis of the informations presented in this short review, a firm conclusion cannot be drawn regarding the pathogenesis of Duchenne muscular dystrophy and more careful fundamental work on the biochemical changes in erythrocyte membranes is likely to be needed.

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