Altered Long Chain Fatty Acids Composition in Duchenne Muscular Dystrophy Erythrocytes

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Abstract. Background: Biochemical abnormalities, increased efflux of soluble enzymes and muscle proteins, and altered permeability of muscle membranes imply the presence of a disorganized erythrocyte membrane in Duchenne muscular dystrophy (DMD). The purpose of the present study was to investigate this hypothesis of a generalized membrane defect. Materials and Methods: Twenty-five patients with the disease were analyzed for their erythrocyte lipid composition and for alterations in their fatty acid content compared to twenty-five healthy subjects. Results: DMD patients showed a decreased concentration of total phospholipids compared to healthy volunteers, with striking fluctuations in concentrations of erythrocyte long chain fatty acids. Specifically, the unsaturated fatty acids such as oleic, linoleic and arachidonic acids were significantly decreased in the disease, whereas the saturated fatty acid, palmitic acid was increased in DMD patients compared to healthy controls. Conclusion: Our findings suggest an abnormal fatty acid composition and disorganization of erythrocyte membrane in patients with DMD associated with possible functional alterations.

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder that usually becomes clinically manifest during the second year of life with musculoskeletal abnormalities (1). It is characterized by chronic degenerative muscle wasting, accompanied by a net negative protein balance and high circulating activities of muscle-derived enzymes such as creatine kinase or aldolase, which are lost from the muscle during the degenerative process (2). Dystrophin has been identified and characterized as the defective gene responsible for the disorder (3). Antibodies raised against part of the protein suggest that it is deficient in Duchenne muscle, whereas in normal muscles dystrophin appears to be localized under the plasma membrane (4). Absence of this protein leads to the muscle degeneration in DMD disorder. The mechanism by which this occurs is currently unknown, although an accumulation of calcium has been implicated in the mechanisms of degeneration of dystrophic muscle (5). There is evidence that calcium mediates the process through activation of calcium-dependent phospholipase enzymes, possibly phospholipase A2, with a consequent release of metabolites of membrane-bound arachidonic acid such as prostaglandins (6).

Furthermore, the drastically elevated activity of creatine phosphokinase in serum suggests a muscle membrane defect which possibly leads to leakage of compounds originally localized in the cytosol of the fibers (7). This hypothesis has been confirmed by the abnormal characteristics of the cell membranes of various cell types in DMD patients and even in a certain proportion of heterozygotes (8). Detailed studies show that in DMD different organ systems are affected, with prominent involvement of cardiographic abnormalities such as cardiac arrhythmias and conduction disturbances (9).

The mechanism involved in the generalized membrane defect, although currently unknown, has been supported by a multiplicity of abnormalities including altered morphology, activity of membrane bound enzymes, permeability and anomalies even in non muscle cells (10-12). More specifically, DMD neutrophils show reduced spontaneous migration and chemotaxis, suggesting that there is a defect in the contractile system or cell membrane functions (13). In addition, DMD fibroblasts showed reduced capping capacity and increased membrane fluidity (14). Nuclear magnetic resonance studies revealed decreased water permeability in DMD erythrocytes, whereas reports on the erythrocyte phospholipids composition of these patients are controversial (15, 16). The fatty acid composition of membrane lipids plays an important role in the physical state of the membrane, which can influence metabolic processes and, therefore, seems an important area of research to test the hypothesis of the generalized membrane defect in DMD patients.
The pellet of erythrocytes was then washed three times with 0.9% NaCl solution in 1.5 EDTA solution in 0.9% v/v NaCl, washed three times with 0.9% NaCl and centrifuged at 12,000 rpm for 20 minutes at 4°C.

Materials and Methods

The erythrocyte fatty acids in total phospholipids fraction were estimated in 25 patients with DMD aged from nine to twenty-two years of age and 25 healthy subjects aged from twelve to thirty-one years old. Diagnosis was based on the conventional criteria (clinical, electromyographic, enzymatic etc.). The patients and the healthy subjects had the same socioeconomic status and approximately the same dietary habits. All reagents used were from Sigma, Europe unless otherwise stated.

Blood samples were drawn early in the morning, after an overnight fasting period, from an antecubital vein and transferred into siliconized capillary tubes. The erythrocytes were isolated by centrifugation overnight. The erythrocytes were then subjected to ultrasonic disintegration and were mixed with a solution of 0.9% NaCl and centrifuged at 9000 rpm for 20 minutes at 4°C. The pellet of erythrocytes was washed three times with 0.9% NaCl solution in 1.5 EDTA solution in 0.9% v/v NaCl, washed three times with 0.9% NaCl and centrifuged at 12,000 rpm for 20 minutes at 4°C (Sorvall RC-2B, Sorvall UK). The pellet of erythrocytes was then analyzed for total lipids as described by Folch et al. (18) employing methanol containing 5% 2, 6-ditert-butyl-b-cresol (BHT) as an antioxidant. Total lipids were further separated into neutral lipids, glycolipids with a mixture of acetone/ methanol 9:1 v/v and phospholipids with a mixture of chloroform/ methanol 2:1 v/v, followed by a definite volume of methanol (about 12 ml). Total phospholipids were estimated by the method of Bartlett (19).

The main fatty acids were determined by gas chromatography in the single erythrocyte phospholipids fraction. The methyl esters of the erythrocyte phospholipids fraction were prepared by conversion of fatty acids to methyl esters as described by Dodge and Phillips (20). The methyl esters were analyzed using gas chromatographic equipment with a flame ionization detector (Perkin-Elmer 900 Gas-Chromatography, Perkin-Elmer Inc., USA) on a column packed with 3% EGS (ethylene glycol succinate) on chromosorb W. The chromatography was isothermal at 180°C. Highly pure nitrogen was used as a carrier gas. Peak identification was made with reference to commercially available methyl esters and the areas of the peaks were measured by triangulation.

All solvents used were redistilled and all experiments were performed in duplicate. Statistical analysis was evaluated by Student’s t-test for unpaired samples. Results are expressed as mean±SD.

RESULTS

Investigation of the erythrocyte membrane lipid composition in patients suffering from DMD compared to normal volunteers led us to determine their fatty acid content as well as their possible role in the overall structure and physiological function of erythrocytes.

Table I. Erythrocyte lipid composition in patients with DMD and healthy subjects.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Controls (N=25)</th>
<th>DMD patients (N=25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total lipids</td>
<td>0.43 ± 0.08</td>
<td>0.41 ± 0.09</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.09 ± 0.007</td>
<td>0.08 ± 0.006</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.006 ± 0.003</td>
<td>0.008 ± 0.003</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>0.33 ± 0.07</td>
<td>0.30 ± 0.07</td>
</tr>
</tbody>
</table>

Values are expressed in mg/10^9 cells (Mean ± SD)

Table II. Percentage concentration of erythrocyte fatty acids in patients with DMD and healthy subjects.

<table>
<thead>
<tr>
<th>Fatty Acids</th>
<th>Controls (N=25)</th>
<th>DMD patients (N=25)</th>
<th>p&lt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
</tr>
<tr>
<td>14:0</td>
<td>2. 80</td>
<td>0.49</td>
<td>3. 40</td>
</tr>
<tr>
<td>16:0</td>
<td>22. 32</td>
<td>1.20</td>
<td>24. 03</td>
</tr>
<tr>
<td>18:0</td>
<td>1. 03</td>
<td>0.34</td>
<td>1. 14</td>
</tr>
<tr>
<td>18:1</td>
<td>18. 10</td>
<td>0.84</td>
<td>17. 24</td>
</tr>
<tr>
<td>18:2</td>
<td>13. 88</td>
<td>0.59</td>
<td>10. 56</td>
</tr>
<tr>
<td>20:4</td>
<td>17. 93</td>
<td>0.81</td>
<td>16. 74</td>
</tr>
<tr>
<td>22:1</td>
<td>1. 66</td>
<td>0.25</td>
<td>1. 78</td>
</tr>
<tr>
<td>24:0</td>
<td>2. 96</td>
<td>0.40</td>
<td>3. 08</td>
</tr>
</tbody>
</table>

Fatty acids are designated by the number of carbon atoms: number of double bonds.

Discussion

Much of the research effort in DMD has been associated with the processes of cytosolic enzyme efflux from the damaged muscle, suggesting altered membrane organization and structure. The involvement of non muscle cells leads to the hypothesis of a generalized membrane functional and

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structural defect, which implicates changes in cellular lipid content and fatty acids composition affecting the activity of cellular enzymic systems.

Concerning the erythrocyte membrane from DMD, abnormalities have been described in a number of their physicochemical properties (21-23). Studies have shown that erythrocytes from Duchenne patients have a lower mechanical resistance, an altered membrane conformation, a higher potassium influx and show a changed electrophoretic mobility (24). In addition, many membrane bound enzymes possess kinetic properties different from control values including acetylcholinesterase, adenylyl cyclase, (Na+ K+) ATPase and protein kinase (25). Furthermore, findings concerning the different membrane lipid classes and, especially, the composition of fatty acids are controversial (15, 16).

In our study, the significant disturbances observed in the erythrocyte long-chain unsaturated fatty acids composition from the disease are of great interest. The significantly decreased concentrations of oleic, linoleic and arachidonic acids indicate changes in the physical state of the membrane, therefore influencing metabolic processes.

Furthermore, the fatty acid composition of the membranes influences their permeability, since increasing unsaturation increases permeability of the cells. It should be noted that all the examined subjects in our study had the same socioeconomic status and the majority of them had similar dietary habits.

Additional evidence suggests that the membrane distortion observed in human muscular dystrophic erythrocytes is related to an abnormal pattern of cation movement and altered functional processes due to changes in membrane lipid content (26, 27). Among the disturbances observed in the concentration of fatty acids in the disease, especially the significant decreases of arachidonic acid (20:4) and linoleic acid (18:2) indicate a tendency for erythrocyte peroxidation. According to Dodge and Phillips (20), the decrease of arachidonic acid is a sensitive index of autoxidation because of its high degree of unsaturation. However, in our study all precautions were taken to minimize oxidation due to experimental procedures. This aspect could be further supported by our control values, which did not show any sign of peroxidation. We, therefore, propose that the decrease of arachidonic acid (20:4) and other unsaturated long-chain fatty acids possibly suggests that the resultant autoxidation predisposes patients with disease to membrane reorganization.

Furthermore, arachidonic acid has been implicated in the hypothesis of an increased calcium-activated phospholipase activity observed in Duchenne muscle. The elevation of intracellular calcium can cause damage to muscle cells. Calcium activation of phospholipase A2 leads to release of arachidonic acid metabolites such as prostaglandins which are further implicated in the mechanism leading to cytosolic enzyme efflux from skeletal muscle (2). In addition, the membrane of Duchenne erythrocytes responds abnormally to high cellular calcium concentrations, which mediate the release of intracellular creatine kinase and, presumably, of other cytoplasmic components.

Also, the change in concentration observed in palmitic acid (16:0) in relation to other alterations plays a fundamental role in the functional alterations of erythrocytes in muscular dystrophies, since the constancy of erythrocyte membrane permeability and fluidity is associated with unchanged lipid content within the species.

We believe that the reduced concentration observed in erythrocyte polyunsaturated fatty acids leads to an increased peroxidation of the cell membrane and altered calcium homeostasis which, in turn, is probably the cause of the perturbation of the whole membrane structure and increased enzymic efflux.

In conclusion, the results of the present study clearly show quantitative changes in the erythrocyte fatty acids from patients with DMD. This may account for the membrane instability and functionality as well as for the disturbed enzymatic transport processes.

References


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