Evaluation of the Intramembrane Particle Count in Biopsied Myocardium from Patients with Idiopathic Cardiomyopathy

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Summary: To clarify the abnormalities of myocardial membrane structure in cardiomyopathy, the density of intramembranous particles (IMP) of mitochondria, sarcoplasmic reticulum and sarcolemma, and the number of caveolar necks was evaluated by freeze-fracture morphology. The subjects consisted of 17 patients with hypertrophic cardiomyopathy (HCM), 6 patients with dilated cardiomyopathy (DCM), and 7 patients as controls. The results showed that the numbers of IMPs on the membrane faces of mitochondria, sarcoplasmic reticulum and sarcolemma were significantly decreased in HCM and DCM, and that they were decreased more markedly in DCM than in HCM. The number of caveolar necks was significantly decreased in DCM. Thus, the decreased numbers of IMPs on myocardial membranes and the decrease in caveolar necks may be important characteristics resulting from abnormal myocardial membrane metabolism in cardiomyopathies, HCM and DCM.

Key words: hypertrophic cardiomyopathy — dilated cardiomyopathy — mitochondria — sarcoplasmic reticulum — sarcolemma — caveolar necks — freeze fracturing — electron microscope

Introduction

Myocardial cells have special membrane structures that act not only as barriers, but also play essential roles in metabolism. Cardiac membranes have two sheets of lipid molecules that form highly ordered structures. The membrane lipid bilayer consists of amphiphilic phospholipid molecules containing both hydrophilic and hydrophobic moieties. In biological membranes, proteins are inserted in the fluid lipid bilayer and the structure and morphology of the membranes are expressed as a fluid-mosaic model (Singer and Nicolson, 1972). The freeze-fracture technique has proven to be one of the most useful methods to study cell membrane structure, and replicas of freeze-fractured membranes contain images of intramembrane particle (IMP) or more complex structures (Rash and Ellisman, 1974).

The cardiomyopathies have been the most difficult of cardiovascular diseases to analyze and understand. The endomyocardial biopsy has been introduced to obtain small specimens from the hearts of patients (Sakakibara and Konno, 1962),

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as direct tissue examination has been a definitive diagnostic method and valuable research tool. Although many approaches, e.g., electron microscopic studies (van Noorden et al. 1971; Baandrup et al. 1981), biochemical studies (Peters et al. 1977; Richardson et al. 1978; Schultheiss et al. 1980) and pharmacologic studies (Fowler et al. 1986) have utilized this material, the pathogenic mechanism of idiopathic cardiomyopathy remains obscure.

Recently it was suspected that membrane damage plays a role in the pathogenesis of cardiomyopathies (Katz et al. 1985; Sakanashi et al. 1991), but no direct morphological evidence of membrane abnormalities of the cardiomyocytes have been reported for this disease.

In this context, freeze-fracture techniques were used to determine whether morphologic abnormalities exist in the membrane structure of human subjects with idiopathic cardiomyopathy.

Materials and Methods

Study group

The subjects included 17 patients with hypertrophic cardiomyopathy (HCM), 6 patients with dilated cardiomyopathy (DCM) and 7 individuals in the control group. The average ages were 45±10 years, 43±11 years, and 48±8 years, respectively. There was no significant difference between the average ages of the three groups. The diagnoses of HCM and DCM were established using the definition and classification by the World Health Organization-International Society and Federation of Cardiology task force (1980). The control group consisted of patients with idiopathic arrhythmia (2 cases), Mobitz type I atrio-ventricular block (2 cases), cured idiopathic myocarditis (1 case), chest pain syndrome (1 case), and mitral prolapse (1 case). The control group had no demonstrative organic disease and no histological abnormalities by light and electron microscopy.

Preparation of the endomyocardial biopsies

The materials were endomyocardial tissues obtained from the right side of the interventricular septum by right ventricular catheterization. After biopsy, the tissues were washed with a 1% potassium chloride solution, immersed immediately in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, at room temperature, fixed for an additional 2 hours at 4°C and transferred to buffer.

Freeze-fracture electron microscopy

After glutaraldehyde fixation, pieces of the tissues were placed in 20% glycerol with 0.1 M cacodylate buffer for 1 hour at room temperature. They were then fitted in the tissue holders and immediately dipped in Freon 22, cooled with liquid nitrogen and froze in liquid nitrogen. The tissue was fractured in an Eiko Engineerings Freeze-Etch Unit (FD-2A) at −115°C, shadowed with platinum and carbon, and replicated with carbon according to the method of Shivers and Brightman (1976). The angle of shadowing in the freeze-fracture were always 60 degrees. The replicas were cleaned with bleach, washed in distilled water and transferred to the 300-mesh copper grids. The fracture faces of the membranes were described using the terminology adopted by Branton (1966).

For transmission electron microscopy, other tissues were postfixed with 1% buffered osmium tetroxide for 90 min at 4°C, and processed in the conventional manner for observation. Sections and replicas were observed with a Hitachi H500 electron microscope.

Quantification of intramembranous particle density

By observation of the replicas, the
number of IMPs, 70 to 100 Å in diameter, per square micrometer was determined. In mitochondria, the particles of the inner protoplasmic face (IP face), inner exoplasmic face (IE face), outer protoplasmic face (OP face) and outer exoplasmic face (OE face) were counted. In sarcoplasmic reticulum and sarcolemma (cardiac plasma membrane), the particles of the P face (adjacent to the cytoplasm) and the E face (adjacent to the extracellular space) were counted. In all instances, care was taken to avoid areas where excessive curvature of the membrane exaggerated the shadowing of the particles. The particle count was determined by magnifying the original negatives, and the final magnification was 66,000×. The counting of the IMPs was performed using the Nexus 6400 Image Processing System (Kashiwagi Research Corp., Tokyo, Japan). At least 100 random portions of the membrane faces of mitochondria, sarcoplasmic reticulum, and sarcolemma on each specimen were evaluated.

Caveolar necks in the sarcolemma of the membrane were counted from low magnification negatives (5,000×) where areas of membrane one square micrometer or greater were fractured.

**Determination of mitochondrial degenerative scores observed by transmission electron microscopy**

For comparison, observations by transmission electron microscopy were made, and the grading of morphological degen-

![Fig. 1](image_url)
eration of mitochondria was determined semi-quantitatively. Degeneration of the mitochondria was graded from 0 to 3. Grade 0 had almost normal mitochondria, grade 1 had slightly damaged mitochondria with a focal sparsity of cristae and mild swelling, grade 2 had moderately damaged mitochondria with a diffuse sparsity of cristae and swelling, and grade 3 had severely damaged mitochondria with destruction of the inner structure (Fig. 1). By evaluating at least 30 random portions of each specimen, the mitochondrial degeneration score was determined for each material.

Statistical analyses
All statistical comparisons were made using the two-tailed Student’s t-test.

Results

Number of intramembrane particles on each intracellular microorganelle

Freeze-fracture replicas clearly distinguished the microstructures in heart muscle, e.g., myofibrils, mitochondria, interstitium, and capillaries (Fig. 2).

Each mitochondrion is bounded by two membranes, thus the replicas of mitochondrion had four fracture faces, two each from the outer and inner membranes (Fig. 3). The number of IMPs of mitochondria was greatest on the IP face of the four faces (Table 1). The mean number on the IP face in HCM was 1,707 per square micrometer, and in DCM was 1,213 per square micrometer, as compared to the control group (C) with 2,137 (HCM

Fig. 2. Freeze-fracture replicas from a control patient exposing large areas of heart muscle cells. Myofibrils, mitochondria, interstitium, and capillaries are clearly distinguished. Mf: myofilaments; Mit: mitochondrion; Cap: capillary; Int: interstitium. Bar=3 μm.
Fig. 3. The P face of an inner mitochondrial membrane (IP) and the E face of an outer mitochondrial membrane (OE) are shown in the upper panel. The E face of an inner mitochondria membrane (IE) and the P face of an outer mitochondrial membrane (OP) are shown in the lower panel. The sample was obtained from a patient with HCM. Bars=0.5 µm (upper panel) and 0.2 µm (lower panel).

vs. DCM vs. C, statistically significant). That is, the mitochondrial particles on the IP face were significantly decreased in HCM and DCM, and they were decreased more markedly in DCM than in HCM. The other faces, that is, the IE face, OP face, and OE face, also had a similar tendency (Table 1).

The number of IMPs in the sarcoplasmic reticulum was larger on the P face than on the E face (Fig. 4), and was significantly decreased in HCM and DCM. The decrease of the IMP in DCM was more prominent than in HCM and control groups (Table 1).

The P face and the E face of cardiac muscle sarcolemma are easily distinguished by the shape of the adjacent caveolae which are either invaginating or protruding (Fig. 5). The numbers of IMPs on sarcolemma were similar to those on sarcoplasmic reticulum. That is, the number of IMPs was larger on the P face than on the E face, and was significantly decreased in cardiomyopathies (Table 1).

Number of caveolar necks
Cardiac muscle sarcolemma contains numerous caveolae (Figs 5 and 6). The number of caveolar necks per square micrometer was significantly decreased in DCM, as compared to both HCM and control groups (Table 2). No significant difference between HCM and the control group was noted.

Mitochondrial degeneration score
The mitochondrial degeneration score was markedly elevated in DCM, and slightly elevated in HCM (Table 3). There was a significant inverse relationship between the mitochondrial score and the IMP density on the IP face (Fig. 7). This means that as degeneration of mitochondria progresses, the number of IMPs decreases.
TABLE 1.  

Number of intramembranous protein particles ( /µm²)

<table>
<thead>
<tr>
<th>P</th>
<th>HCM</th>
<th>DCM</th>
<th>C</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HCM vs. C</td>
<td>DCM vs. C</td>
<td>HCM vs. DCM</td>
<td></td>
</tr>
<tr>
<td>Mitochondria</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IP face</td>
<td>1707±157</td>
<td>1213±170</td>
<td>2137±237</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IE face</td>
<td>881±190</td>
<td>508±118</td>
<td>1234±246</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>OP face</td>
<td>487±66</td>
<td>273±33</td>
<td>630±112</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>OE face</td>
<td>295±26</td>
<td>190±34</td>
<td>340±107</td>
<td>NS</td>
</tr>
<tr>
<td>Sarcoplasmic Reticulum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P face</td>
<td>1621±185</td>
<td>1189±160</td>
<td>1937±286</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>E face</td>
<td>292±64</td>
<td>201±58</td>
<td>415±78</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sarcolemma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P face</td>
<td>1629±224</td>
<td>1071±203</td>
<td>1865±254</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>E face</td>
<td>207±34</td>
<td>138±26</td>
<td>251±41</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Values are mean±SD;  HCM: hypertrophic cardiomyopathy;  DCM: dilated cardiomyopathy;  C: control;  P: protoplasmic;  E: exoplasmic;  IP: inner protoplasmic;  OP: outer protoplasmic;  IE: inner exoplasmic;  OE: outer exoplasmic.

Fig. 4. The E face (left panel) and the P face (right panel) of cardiac muscle sarcolemma demonstrate populations of IMPs. On the E face, the adjacent caveolae is protruding (left upper part), while on the P face, it is invaginating (right upper part). The intramembranous particles on the P face of the sarcolemma are numerous, as compared to the E face. The sample was obtained from a patient with HCM. Bar=0.1 µm.
Fig. 5. Higher magnification replicas of the P and E faces of the sarcoplasmic reticulum. The intramembranous particles on the P face of the sarcoplasmic reticulum are numerous, as compared to the E face. The sample was obtained from a patient with DCM. Bar=0.2 μm.

**TABLE 2.**

*Number of caveolar necks ( /μm²*)

<table>
<thead>
<tr>
<th></th>
<th>HCM</th>
<th>DCM</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sarcolemmal</td>
<td>14.2 ±1.6*</td>
<td>10.5 ±2.6*</td>
<td>14.3 ±2.2</td>
</tr>
<tr>
<td>Membrane</td>
<td></td>
<td></td>
<td></td>
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</table>

Values are mean ± SD: HCM: hypertrophic cardiomyopathy; DCM: dilated cardiomyopathy; C: control; Statistical p-values: *p < 0.001 (HCM vs. DCM); #p < 0.05 (C vs. DCM)

**TABLE 3.**

*Mitochondrial degeneration score*

<table>
<thead>
<tr>
<th></th>
<th>HCM</th>
<th>DCM</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Score</td>
<td>0.99 ±0.15*</td>
<td>2.24 ±0.26*</td>
<td>0.45 ±0.27*</td>
</tr>
</tbody>
</table>

Values are mean ± SD: HCM: hypertrophic cardiomyopathy; DCM: dilated cardiomyopathy; C: control; Statistical p-values: *p < 0.001 (HCM vs. DCM vs. C)
Fig. 6. A low magnification replica of cardiac muscle sarcolemma from a HCM patient (same patient as in Fig. 5). The upper panel shows the E and P faces, and the lower panel shows the P face. The cardiac muscle sarcolemma contains numerous caveolae. Bars=0.2 μm.
Fig. 7. Inverse relationship between the mitochondrial degeneration score and the mitochondrial IMP density on the IP face.

Discussion

Although various electron microscopic techniques were applied for the ultrastructural characterization of human heart muscle cells in biopsy material (Dalen et al. 1987), no previous studies on cardiac muscle membrane structures using freeze fracturing in patients with idiopathic cardiomyopathy have been reported. The present findings show that morphological changes do occur within mitochondrial membranes, sarcolemmal membranes, and membranes of sarcoplasmic reticulum in cardiomyopathies. The densities of intramembranous particles are slightly decreased in HCM and markedly decreased in DCM, as compared to controls. On electron microscopic examination DCM had a high degree of degenerative mitochondrial changes, which were consistent with the marked reduction of the myocardial intramembranous particles. These particles have been interpreted to be proteinaceous like receptors (Luther and Peng, 1982), pumps (Kordylewski et al. 1983), general membrane proteins (Marchesi et al. 1973; Elgsaeter and Branton, 1974), enzymes (Tillack et al. 1972; Akiyama et al. 1985; Sjoestrand and Candipan, 1985), etc. Therefore, it is possible that this reduction in the intramembranous particles in each membrane is indicative of some membranous enzymatic abnormality or dysfunction of the microorganelles in cardiomyopathies.

Experimentally the diminution of IMPs in heart muscle is found with the conditions of anoxia and ischemia. Frank et al. (1988) reported that severe anoxia significantly decreased (31%) the intramembranous particles in the P face of the membrane and in the E face (25%). Ashraf (1978) reported that myocardial ischemia induced alterations in lipid fluidity and the distribution of IMPs in the mitochondrial membranes. Moreover, ischemia-induced IMP diminution was observed in cardiac sarcoplasmic reticulum (Imai et al. 1983; Akiyama et al. 1985). Changes of IMPs in the cardiac muscle cell plasma membrane were observed in adriamycin-induced cardiotoxicity (Severs et al. 1991). These reports indicate that various secondary stresses can induce membrane changes of microorganelles in cardiomyocytes.

Membrane changes were observed in hereditary cardiomyopathic hamsters, leading to myocardial hypertrophy and progressive heart failure, and this model provided much morphological and physiological information. Graham et al. (1984) reported that up to the age of 1 month, the particle density was the same in two strains of hamsters (Bio 14.6 and normal randombred). After this time, the number of IMPs was about 30% lower in dystrophic than in normal heart sarcolemma. The same alterations are found in the skeletal muscle cell plasma membrane in the dystrophic mouse (Shivers and Atkinson, 1984) and human Duchenne muscular dystrophy (Schotland et al. 1977; Schotland et al. 1981; Bonilla et al. 1983). On the other hand, Berry et al. (1983) quantified the densities of IMPs in sarcolemmal membranes from the ven-
tricular myocardium of 25 day-old U.M.-X 7.1 cardiomyopathic Syrian hamsters. They reported that the IMP numerical densities were significantly increased above control values in the cardiomyopathic hamsters. The reasons for the difference are not clear. An increase of sarcolemmal protein(s) may appear at some stage as an adaptive process in this genetically defective animal.

Interestingly, this study demonstrates that numerical densities of IMPs in HCM are slightly decreased in all cardiac membranes. A morphometric analysis of transmission electron micrographs verified that mitochondria in cardiac muscle with HCM had increased sizes and numbers (data not shown). The significance of this finding is not known. Ozawa et al. (1990) reported that mitochondrial abnormalities were present in mitochondrial DNA in patients with HCM. Thus, it is suspected that the function of a mitochondrion per se in the cardiomyocyte in HCM is impaired. The mitochondriosis found in HCM may be an adaptive reaction to maintain sufficient energy production in the hypertrophic cardiomyocyte. Further studies are necessary to clarify whether the diminution of IMPs is a primary defect in HCM.

As the density of the IMPs in cardiac membranes vary by species, organelles, and freeze-fracture procedures, it is hard to obtain an absolute number for the IMPs in each membrane. The P-face particle densities of the plasma membrane from myocardial cells of mammalian ventricular heart muscle are 1,400-2,300 per square micrometer (Frank et al. 1980; Kordylewski et al. 1983). Data from cardiac biopsies showed 1,865 ± 254 particles per square micrometer on the P face and 755 ± 171 particles per square micrometer on the E face in the sarcolemma, which was lower than that for normal human biceps (Bonilla et al. 1983). With mitochondria, the IMP number for the inner membrane was almost the same as that for the dog (Ashraf, 1978). Anyway, the number of IMPs in the human myocardium obtained in the present study do not demonstrate big differences among mammalian species.

The number of caveolae in freeze-fracture preparations of cardiac muscle plasma membrane of patients with HCM and DCM and of control subjects were analyzed. The results have shown a significant decrease in the number of caveolae in cardiac muscle plasma membranes from a population of patients with DCM. The physiological role of the caveolae has not been fully elucidated, but three hypotheses have been considered. 1) The caveolae are a membrane reservoir for stretch (Prescott and Brightman, 1976). 2) The caveolae are part of the transverse tubular system (Ezerman and Ishikawa, 1967; Franzini-Armstrong et al. 1975; Zampighi et al. 1975; Forbes et al. 1984). 3) The caveolae are pinocytotic vesicles (Banker et al. 1979). Whether the decrease in caveolar density noted in DCM is also associated with a loss of the transverse tubular system or a depressed pinocytotic function is unknown. It is possible that the changes in numbers of caveolae in DCM observed in this study are related to degeneration of the cardiac muscle, since a diminution of intramembranous particles and a high mitochondrial degeneration score by electron microscopy were observed in this disease group.

Freeze-fracture studies of experimentally designed cardiac muscle degeneration at different stages will yield further insight into this possibility. New technologies in this field will make it possible to identify the nature of each IMP, directly (Quick and Letourneau, 1988).
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**References**


Rec. 202, 116A-117A.


