STUDIES ON ULTRASTRUCTURE AND CYTOCHEMICAL ATPase ACTIVITY IN HUMAN CARDIAC MYOCYTES FROM BIOPSIES FROM PATIENTS WITH VARIOUS HEART DISEASES

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YOUICHI TAKEYAMA, M.D., AND TAKASHI KATAGIRI, M.D.

Ultrastructural localization and intensity of ATPase activity were studied in myocardial cells from biopsies with reference to fine-structural alterations and cardiac functions in patients with various heart diseases.

ATPase activity was found to be intense in the sarcoplasmic reticulum (SR), the matrices of the mitochondria (Mt), on the myofilaments (Mf) and along the gap-junctions of intercalated discs in the control myocardial cells. ATPase activity was more intense in cardiac myocytes from well-functioning or ultrastructurally well preserved hearts. In failing and degenerating hearts, ATPase activity was decreased. ATPase activity was more intense in clinically-improving than in clinically-worsening patients. However, the localized pattern of ATPase activity was similar in each heart disease.

These results suggest that cytochemical observation of ATPase activity can reflect not only fine structural changes in cardiac myocytes, but also the metabolic state in the diseased heart, and is valuable therefore from the standpoint of clinical medicine.

ATPase is one of the essential enzymes in energy metabolism of heart muscle and is well known to be intimately related to myocardial function. There is controversy as to whether ATPase activity can decrease in the failing heart both in experimental animals and in humans1–6 and few cytochemical studies are available on ATPase activity of human myocardium7–11. The present study was designed to assess the relationship between fine-structural changes and ATPase activity in patients with various heart diseases. We performed endomyocardial biopsy at catheterization in patients with various heart diseases and investigated ATPase activity as an index of cellular metabolism, together with fine structural changes, under an electron microscope.

PATIENTS AND METHODS

Myocardial biopsy: Endomyocardial biopsy was carried out in 74 patients, 40 men and 34 women, ranging in age from 16 to 73 (average age 45). We explained adequately the necessity and safety of the biopsy procedure to each patient, and obtained his or her consent. Their profiles are listed in Table I. The clinical course of the patients and intensity of ATPase activity are summarized in Table II. The patients were divided into three groups, according to their clinical course, the first worsened clinically and hemodynamically, i.e. precipitation of the

Key words:
Human myocardial biopsy
Electron microscopy
ATPase activity

(Received September 18, 1986; accepted April 27, 1987)
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subjective symptoms, increase in the cardiothoracic ratio (CTR) on the chest X-ray film and aggravation of the physical findings, the second improved and the third unchanged in these respects. As representative parameters indicating cardiac function, cardiac index (CI), ejection fraction (EF), left ventricular end-diastolic pressure (LVEDP), and pulmonary capillary wedge pressure (PCWP) were measured at the cardiac catheterization. Cardiac specimens were obtained, utilizing a Machida-type biotome, from both ventricles in most of the patients.

Cytochemical determination of ATPase activity: Specimens were immediately prefixed with 0.25% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate (pH 7.4) at 4°C for 20 min, rinsed in 0.1 M sodium cacodylate (pH 7.4) three times and cut into 40 μm thick frozen sections at −30°C with a cryostat. The thin sections were rinsed in 0.1 M tris-maleate (pH 7.4) and incubated in the modified solution of Wachstein-Meisel12 for ATPase reaction at 37°C for 60 min. The solution consisted of 4 mM ATP as a substrate, 3 mM Pb (NO₃)₂, 20 mM CaCl₂, 20 mM MgSO₄, 0.1 M tris-maleate (pH 7.4) and 5% dimethyl sulfoxide (DMSO).

After incubation, sections were rinsed briefly in 0.1 M tris-maleate (pH 7.4), postfixed in 1% OsO₄ at 4°C for 60 min, and embedded in Epon 812, after dehydration through a graded series of ethanol and propylene oxide. Ultra thin sections were cut by a Sorvall Porter-Blum ultramicrotome, MT-2, and double stained with uranyl acetate and lead citrate, and observed with Hitachi HS-9 and H-300 electron microscopes. We usually observed ten myocardial cells for the estimates of ATPase activity and morphological findings.

As control experiments, the reaction was also performed in ATP-free solution, or in a reaction media with addition of 1 mM L-bromotetramisole as an inhibitor of non-specific alkaline phosphatase, or with the addition of 0.1 mM ouabain as an inhibitor of Na⁺-K⁺ ATPase.

Grading of cytochemical ATPase activity and statistical analysis: The intensity of cytochemical ATPase activity in SR, in M₁ and on M₂ was graded on the micrographs into 4 degrees classified as negative, weak, moderate and intense.

Before evaluating the relations between ATPase activity and cardiac index, we examined the distribution of the observations of CI by fitting the power-normal distribution to them. As a result, the power transforming parameter (λ) was estimated as λ = −0.24. Then, a likelihood ratio test was performed under null hypothesis H₀: λ = 0.0, which corresponds to log transformation. The significance probability of the test was 0.84, so that the hypothesis was not rejected and the CI was assured to be a log normal distribution. Therefore, we subsequently used log (CI) in the analysis. The relations between ATPase activity and log (CI) were evaluated by simple regression analysis, using the log (CI) as dependent variable and ATPase activity at the time as independent variable. A Mann-Whitney U-test was performed to determine the degree of difference in ATPase activity between the patients who improved clinically and those who worsened.

RESULTS

1. Control myocardial cells: Fig. 1 shows electron micrographs of cytochemical ATPase

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**TABLE 1 DIAGNOSES OF HEART DISEASES IN 74 PATIENTS**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Valvular disease</td>
<td></td>
</tr>
<tr>
<td>Mitral valvular disease</td>
<td>9</td>
</tr>
<tr>
<td>Aortic valvular disease</td>
<td>2</td>
</tr>
<tr>
<td>Combined valvular disease</td>
<td>3</td>
</tr>
<tr>
<td>Cardiomyopathy</td>
<td></td>
</tr>
<tr>
<td>Hypertrophic cardiomyopathy</td>
<td>6</td>
</tr>
<tr>
<td>Dilated cardiomyopathy</td>
<td>11</td>
</tr>
<tr>
<td>Myocarditis</td>
<td>6</td>
</tr>
<tr>
<td>Atrial septal defect</td>
<td>1</td>
</tr>
<tr>
<td>Ventricular septal defect</td>
<td>1</td>
</tr>
<tr>
<td>Primary pulmonary hypertension</td>
<td>1</td>
</tr>
<tr>
<td>Hypertensive heart disease</td>
<td>7</td>
</tr>
<tr>
<td>Ischemic heart disease</td>
<td>5</td>
</tr>
<tr>
<td>Amyloidosis</td>
<td>2</td>
</tr>
<tr>
<td>Arrhythmia</td>
<td>17</td>
</tr>
<tr>
<td>Neurocirculatory asthenia</td>
<td>3</td>
</tr>
</tbody>
</table>


*Japanese Circulation Journal Vol. 31, November 1987*
TABLE II  CLINICAL FEATURES OF PATIENTS AND INTENSITY OF ATPase ACTIVITY OF BIOPSIED MYOCARDIAL CELLS

<table>
<thead>
<tr>
<th>Clinical Course</th>
<th>Case</th>
<th>Age</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>NYHA</th>
<th>CTR %</th>
<th>CI l/min/m²</th>
<th>LVEDP mmHg</th>
<th>ATPase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Improved</td>
<td>I.K.</td>
<td>36</td>
<td>F</td>
<td>MS</td>
<td>I</td>
<td>63−58</td>
<td>1.93</td>
<td>2</td>
<td>++ + ++</td>
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<tr>
<td></td>
<td>Y.I.</td>
<td>62</td>
<td>F</td>
<td>MS</td>
<td>II→I</td>
<td>69−66</td>
<td>1.96</td>
<td>14</td>
<td>+ + +</td>
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<tr>
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<td>36</td>
<td>F</td>
<td>MS</td>
<td>III→I</td>
<td>61−47</td>
<td>2.70</td>
<td>11</td>
<td>+++ +++ ++</td>
</tr>
<tr>
<td></td>
<td>Y.A.</td>
<td>59</td>
<td>F</td>
<td>MS</td>
<td>II→I</td>
<td>69−62</td>
<td>1.88</td>
<td>6</td>
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</tr>
<tr>
<td></td>
<td>K.E.</td>
<td>41</td>
<td>F</td>
<td>MS, ASR</td>
<td>I</td>
<td>76−71</td>
<td>2.33</td>
<td>18</td>
<td>++ + +++</td>
</tr>
<tr>
<td></td>
<td>Y.N.</td>
<td>44</td>
<td>F</td>
<td>MSR, AS</td>
<td>II→I</td>
<td>66−63</td>
<td>2.19</td>
<td>7</td>
<td>+++ + +++</td>
</tr>
<tr>
<td></td>
<td>T.S.</td>
<td>39</td>
<td>M</td>
<td>MR</td>
<td>IV→II</td>
<td>58−47</td>
<td>2.80</td>
<td>18</td>
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</tr>
<tr>
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<td>M</td>
<td>MR</td>
<td>II→I</td>
<td>56−50</td>
<td>2.77</td>
<td>+++</td>
<td>+++ ++</td>
</tr>
<tr>
<td></td>
<td>T.O.</td>
<td>51</td>
<td>F</td>
<td>MSR, ASR</td>
<td>II→I</td>
<td>68−60</td>
<td>1.99</td>
<td>+</td>
<td>+ +</td>
</tr>
<tr>
<td></td>
<td>C.T.</td>
<td>48</td>
<td>F</td>
<td>HCM</td>
<td>II→I</td>
<td>60−53</td>
<td>3.69</td>
<td>22</td>
<td>+++ ++ +++</td>
</tr>
<tr>
<td></td>
<td>U.M.</td>
<td>73</td>
<td>M</td>
<td>HCM</td>
<td>I</td>
<td>59−54</td>
<td>2.92</td>
<td>18</td>
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<tr>
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<td>F</td>
<td>DCM</td>
<td>I</td>
<td>59−56</td>
<td>3.05</td>
<td>16</td>
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</tr>
<tr>
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<td>27</td>
<td>F</td>
<td>DCM</td>
<td>II→I</td>
<td>52−41</td>
<td>4.29</td>
<td>10</td>
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<tr>
<td></td>
<td>T.S.</td>
<td>36</td>
<td>M</td>
<td>DCM</td>
<td>I</td>
<td>57−51</td>
<td>3.31</td>
<td>11</td>
<td>+++ ++</td>
</tr>
<tr>
<td></td>
<td>T.O.</td>
<td>57</td>
<td>F</td>
<td>DCM</td>
<td>III→I</td>
<td>64−50</td>
<td>2.54</td>
<td>17</td>
<td>++ ++ ++</td>
</tr>
<tr>
<td></td>
<td>S.A.</td>
<td>42</td>
<td>M</td>
<td>DCM</td>
<td>II→I</td>
<td>56−43</td>
<td>2.51</td>
<td>19</td>
<td>++ + ++</td>
</tr>
<tr>
<td></td>
<td>N.N.</td>
<td>60</td>
<td>M</td>
<td>DCM</td>
<td>IV→II</td>
<td>62−54</td>
<td>2.83</td>
<td>18</td>
<td>++ +++ ++</td>
</tr>
<tr>
<td></td>
<td>H.N.</td>
<td>56</td>
<td>M</td>
<td>DCM</td>
<td>I</td>
<td>59−53</td>
<td>2.86</td>
<td>11</td>
<td>+++ ++ ++</td>
</tr>
<tr>
<td></td>
<td>M.K.</td>
<td>17</td>
<td>F</td>
<td>Myocarditis</td>
<td>I</td>
<td>42</td>
<td>3.20</td>
<td>+</td>
<td>++ +</td>
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<tr>
<td></td>
<td>N.H.</td>
<td>66</td>
<td>F</td>
<td>A-V Block</td>
<td>II→I</td>
<td>58−52</td>
<td>1.75</td>
<td>13</td>
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</tr>
<tr>
<td></td>
<td>M.Y.</td>
<td>58</td>
<td>F</td>
<td>A-V Block</td>
<td>II→I</td>
<td>59−48</td>
<td>3.36</td>
<td>12</td>
<td>+ + ++</td>
</tr>
</tbody>
</table>

| Worsened        | S.A. | 60  | F   | MSR      | I    | 63−68 | 2.14      | 15        | + + +         |
|                 | K.K. | 37  | F   | MSR      | I→II | 56−62 | 1.98      | 14        | + − +         |
|                 | N.N. | 59  | M   | DCM      | II→IV| 51−62 | 1.67      | 18        | + + −         |
|                 | T.W. | 68  | M   | DCM      | II→III| 60−66 | 2.45      | 15        | + + +         |
|                 | M.K. | 17  | F   | Myocarditis| I | 42     | 3.10      | − +        |              |
|                 | E.S. | 45  | F   | Amyloidosis| I→III| 61−68 | 3.36      | 14        | + + +         |
|                 | M.S. | 32  | M   | Amyloidosis| I    | 33     | 3.47      | 14        | ++ + +        |

Abbreviations are used as follows: CTR cardiothoracic ratio: CI cardiac index: LVEDP left ventricular end-diastolic pressure: MS mitral stenosis: MSR mitral stenosis and regurgitation: AS aortic stenosis: ASR aortic stenosis and regurgitation: HCM & DCM hypertrophic and dilated cardiomyopathy: A-V Block atrioventricular block.

activity in the myocardial cells, which were regarded as normal control cells due to the absence of abnormal findings by the various examinations and fine-structural observations. As briefly illustrated in our previous report,13 the reaction product of ATPase activity was observed to be intense in terminal cisternae (TC) of the SR and matrices of the Mt, moderate on the Mt, along the cytoplasmic side of gap-junctions of intercalated discs, in subsarcolemmal cisternae and in longitudinal tubules of the SR (Fig. 1-A, B and C). ATPase activity was also recognized in vascular endothelial cells and in the outer membrane of red blood cells (Fig. 1-D). However, no reaction products were found either in the nuclei, sarclemma or lysosomes.

In control experiments in which ATP-free medium was used, almost no reaction product
was found. In other control experiments in which an incubation medium with L-bromotetramisole or ouabain was used, reaction product was recognized to be as intense as that with the original incubation medium.

2. Diseased myocardial cells: Cellular changes in the diseased myocardial cells were not homogeneous. Therefore, although morphological observation should be made of as many myocardial cells as possible, it was impossible to adequately observe enough of these, because the biopsies were extremely small (1–3 mg) and cytochemical analysis on enzymatic activity was difficult to carry out. For this reason, about ten myocardial cells were observed for each human myocardial biopsy.

In diseased myocardial cells, ultrastructural alterations were noted, and ATPase activity was decreased markedly in the cells with severe ultrastructural changes. Fig. 2-A shows a myocardial cell in which ATPase activity markedly decreased in the swelling or disrupted SR. The presence of a mitochondrial population with abnormal variations in size was observed, and in places ATPase activity was decreased. Fig. 2-B shows a myocardial cell with severe degenerative mitochondrial changes, such as swelling and partial or complete dissolution of cristae, and with markedly decreased ATPase activity. Similarly ATPase activity evidently decreased on Mf with coarsening and Z-band abnormality. Fig. 2-C shows a myocardial cell, with derangement and brush-like formation of Mf, combined with loss of Z-band material, lack of ordinary structures of the sarcomere and almost no ATPase activity on Mf.

3. Case presentation: We carried out biopsies twice in two clinically improved cases and found that ATPase activity was more intense after recovery.

Case N.N., a 59-year-old man, had been drinking 900 ml of Japanese sake daily for 35 years. He began, insidiously, to become slightly short of breath five years ago and cardiac failure had been repeatedly diagnosed. He was admitted to our hospital because of dyspnea at rest. CTR was 62% on chest X-ray and CI was 1.67 l/min/m². ATPase activity was generally weak in SR, in Mt and on Mf (Fig. 3-A). When he recovered clinically about 13 months after the first biopsy, ATPase activity was observed to be much higher in Mt and SR (Fig. 3-B).

Case M.K., a 17-year-old female, began to complain of fever and palpitation three weeks before admission. Frequent attacks of paroxysmal supraventricular tachycardia (PSVT) were observed. She was diagnosed by endomyocardial biopsy to have acute myocarditis. The reaction product of ATPase activity was reduced in disrupted Mt and on interrupted Mf (Fig. 4-A). Two months later it was markedly recovered in both Mt and SR, when she had recovered (Fig. 4-B).

4. Correlation of ATPase activity and cardiac function: Fig. 5 shows the correlation between ATPase activity of each micro-organ, CI and clinical course. In SR, the regression line was $Y = 0.86 + 0.14X$, the significance probability (p) was 0.001 and the proportion of variation explained (PVE), by the regression, was 0.131. In Mt, the regression line was $Y = 0.91 + 0.13X$, $p = 0.001$ and PVE = 0.127. Similarly in Mf, the regression line was $Y = 0.89 + 0.14X$, $p = 0.001$ and PVE = 0.144. From these results, a signifi-
Fig. 3. Electron micrographs showing ATPase activity in the myocardial cells of alcoholic
dilated cardiomyopathy. A bar indicates 1 μm.
A: ATPase activity was generally weak in SR, Mt and on Mf when the patient had
severe heart failure.
B: ATPase activity was observed much higher when the patient had clinically
recovered.

Fig. 4. Electron micrographs showing ATPase activity in the myocardial cells of acute
myocarditis. A bar indicates 1 μm.
A: ATPase activity was slightly observed in disrupted Mt and on interrupted Mf
when the patient deteriorated.
B: ATPase activity was markedly increased when the patient recovered.

cient correlation was noted between ATPase activity of each micro-organ and CI. It appeared
that ATPase activity was more intense in patients with better cardiac function. In the patients
who had improved high ATPase activity was found, even though CI was low, as is shown in
the lower right of Fig. 5. In the patients who had worsened ATPase activity was low, as is
shown in the lower left of Fig. 5. In all sites, a higher level of ATPase activity was found in
the patients who had improved. The difference is statistically significant from the patients who had
worsened (p < 0.05 Mann-Whitney U-test).

Higher ATPase activity was seen in ultrastructurally well preserved myocardial cells, in well
functioning hearts and in patients who had recovered both clinically and hemodynamically.
There were no specific changes in localization of ATPase activity in each heart diseases and the
intensity of ATPase activity was similar in both ventricles of the same patient.

DISCUSSION
ATPase is one of the essential enzymes in vital phenomena, and it is conceivable that the impair-
ment of its activity in cells makes it difficult for them to function properly. Therefore it is very
important to study the ATPase activity of cardiac myocytes in various heart diseases.

*Japanese Circulation Journal  Vol. 51, November 1987*
ATPase activity of the diseased heart has been studied mainly from the biochemical viewpoint of the isolated myocardial fractions such as contractile proteins, mainly myosin, SR, sarcolemma and Mt, in cardiac hypertrophy, ischemic heart disease and thyrotoxic heart disease. In such studies, however, fine structural changes in the cardiac myocytes have not been discussed in relation to altered ATPase activity.

It is evident that excessive fixation may significantly reduce enzymatic activity and that insufficient fixation may not maintain the fine structure of cell organelles. In this study we used as the fixative for ATPase activity, a combination of 0.25% glutaraldehyde and 2% paraformaldehyde in cacodylate buffer, to gain the best result. This fixative gave good maintenance of ultrastructure of the myocardial cells and fair preservation of cytochemical ATPase activity.

Localization of ATPase activity in a cardiac myocyte has already been fully established in SR, sarcolemma, Mt and Mf. The method of Wachstein and Meisel has been used widely for cytochemical detection of ATPase activity. In this method lead nitrate is utilized to precipitate the reaction product, demonstrating ATPase activity as a form of lead phosphate, and there has been much discussion about the intensity and localization of ATPase in animal cardiac myocytes.

In the present study we modified the original method of Wachstein and Meisel. We reduced the amount of lead nitrate to 3 mM, but increased the content of ATP to 4 mM and added 5% DMSO to the fixative and the incubation medium, to enhance the membrane permeability of substrate into tissues, and to preserve the ultrastructure of cell organelles in cardiac myocytes. As a result of these modifications, we showed the reaction product not only in SR and Mf but also in Mt. The pattern of localization and intensity of ATPase activity in normal human cardiac myocytes was similar to that reported by Sommer and Spach, Ferrans et al., Ogawa and Mayahara and Ozawa et al., all of whom used animal heart muscle. Moreover by the addition of DMSO, the reaction product was shown to be intense in the matrix of Mt. The reaction product filled the matrices, while cristae were entirely devoid of reaction product, probably because of protrusion of elementary particles attached to the inner membrane, representing the site of localization of ATPase.

Fig.5. Correlation of ATPase activity, cardiac index and clinical course in various heart diseases. Significant correlation was noted between the intensity of ATPase activity and cardiac index (p < 0.001). Open circles, solid circles and crosses correspond to improved, unchanged and patients clinically worsened. Dashed line corresponds to the regression line.
into matrices.\textsuperscript{23}
We used ATP as a substrate as a matter of

course, but ATP can be hydrolysed not only by

ATPase but also by non-specific phosphatases.

In order to check the specificity of the reaction

product, an incubation medium with 1 mM L-
bromotetramisole and 0.1 mM ouabain as inhibi-
tors of non-specific alkaline phosphatase and Na\textsuperscript+-
K\textsuperscript{+} ATPase, respectively, was used in control

experiments. The ATPase activity was found to

be almost as intense as when the original incuba-

tion medium was used.

As ATPase activity of the diseased heart

muscle has mainly been studied from the

biochemical viewpoint by extracting the micro-

organs, which include ATPase protein, the

material used was limited to that from animals

or cadavers. In hypertrophic and failing hearts,

a 15\% to 20\% reduction in myosin ATPase

activity was noted and recently a change in

myosin isozymes, i.e. from the alpha to the beta

type myosin molecule, has been established.\textsuperscript{25} In

acute ischemic myocardial necrosis, degradation

of ATPase proteins of sarcolemma\textsuperscript{14} and SR\textsuperscript{15} in

the early stage of ischemic injury has been

observed, followed by the degradation of myo-

sin\textsuperscript{20} in which cytochemical ATPase activity was

shown to decrease simultaneously with ischemic

fine structural changes.\textsuperscript{24}

In cytochemical studies on ATPase activity

cardiac myocytes, it should be possible to

observe the intensity of ATPase activity quanti-

tatively in localized areas of diseased human

heart muscle cells. However it was very difficult

to detect detailed changes in ATPase activity

using this method. In this respect, little work

has been carried out on cytochemical enzymatic

activity in the diseased myocardial cells\textsuperscript{7,9,10,13}

and controversy continues as to whether ATPase

activity is correlated to cardiac pathological

states and functions.\textsuperscript{3,4,18,26,27}

We observed a reduction in the ATPase reaction

product in cells with clear ultrastructural

changes such as swelling and disruption of SR,

swelling and dissolution of Mt, and overstretch,

interruption and disarrangement of My. The

reduction of ATPase activity in accordance

with clear ultrastructural changes is assumed to be due
to the alteration of the ATPase protein molecule

itself.

In this study specific changes in ATPase

activity related to each heart disease were not

found. Instead, the activity was roughly corre-

lated with the metabolic activity of the myocar-
dial tissue. In well-functioning hearts, more

intense activity was found, and vice versa. The

reason has not yet been fully elucidated, and

may represent the conformational changes in

ATPase molecules\textsuperscript{25,29} or may be caused by

alterations in the intracellular fine structure

which influence the way ATPase works. This

phenomenon was observed in cardiac myocytes

whose fine structures were relatively well re-
tained as opposed to cells whose fine structures

were severely damaged where ATPase activity

was reduced to a great extent. In the latter case,

the degradation of ATPase protein is presumed
to have taken place. It is well documented that

there are good correlations between the morphol-

ogy of cardiac muscles as obtained from

biopsies to cardiac functions and to the prognosis

of the patients.\textsuperscript{30,31}

In this study, lower ATPase activity was

observed in the group of patients who deterio-

rated clinically. On the contrary ATPase activity

tended to be more intense in well-functioning

hearts and in clinically improved patients. These

facts indicate that the level of cytochemical

ATPase activity of heart muscle cells is not

constant under the same disease conditions, but

varies in proportion to the clinical, i.e. metabolic

state of the patient. This is very important in

clinical practice.

Changes in the level of ATPase activity may

be affected not only by morphologic alterations

in the cardiac myocytes, but also by many

factors which qualify their activities such as the

catecholamine-cyclic AMP system and the new

synthesis of myosin isozymes with low ATPase

activity.

We conclude that cytochemical detection of

ATPase activity shows the metabolic activity of

diseased cardiac myocytes from biopsies and is

very useful in combination with fine-structural

findings, in determining not only the pathologic

state, but also the metabolic state of the heart

muscle cells.

\textbf{Acknowledgements}

\textit{The authors wish to express sincere thanks to

Professor Hirokazu Nittani and Professor Yasumitsu

Nakai for their kind guidance and encouragement

during this work, and they are indebted to Drs. Mikihiko

Yokoyama, Suniyasu Sekita and Fumihiro Tanno for

their collaboration.}

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