HISTOCHEMICAL, ULTRASTRUCTURAL AND CYTOCHEMICAL STUDY OF REPERFUSION EFFECT ON ISCHEMIC MYOCARDIAL INJURY

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We assessed the histochemical, ultrastructural and cytochemical effects of reperfusion on ischemic myocardial cells during the early and late reperfusion phases in two groups of dogs. Group A were 8 dogs undergoing 1 hour occlusion of LAD, and Group B were 14 dogs undergoing 1 hour occlusion of LAD followed by 2 hour reperfusion period. The results of the histochemical study (PAS stain) demonstrated that in Group A, a patchy distribution of glycogen occurred primarily in the subepicardial region. Three-dimensional analysis of this distribution revealed peninsulas of glycogen running parallel with a vessel. The cells in Group B, mainly subepicardium, showed a moderate glycogen content which was more extensive than those in Group A. The ultrastructural changes were assessed after a 60-minute ischemia and subsequent recovery (after 5 minutes and 120 minutes of reflow) using transmural biopsy specimens. Each myocardial cell was graded from 0-4 according to the degree of ischemic injury and recovery. The degree of ischemic damage varied in intensity from slight to severe, in both the subepicardium and the subendocardium. Ca**-ATPase activity was examined cytochemically in myocardial cells of Group B. After 60-minute occlusion, the moderately ischemic cells (especially in the subepicardium) that were without amorphous dense bodies or marked sarcocissial lifting-off made significantly greater ultrastructural recovery (p < 0.05) with restoration of Ca**-ATPase activity on sarcoplasmic reticulum and mitochondria after 120 minutes of reflow. This occurred even though after 5 minutes of reflow the cell showed temporary deterioration such as contraction bands, vacuoles and severe destruction of some mitochondria.

MANY investigators have described the phenomena associated with reperfusion after transient coronary artery occlusion, and the revascularization produced by the intracoronary thrombolytic method or surgical bypass operation in patients with acute myocardial infarction. Some myocardial cells are irreversibly injured by transient ischemia. On the other hand, although short periods of ischemia produce metabolic, functional and ultrastructural changes, restoration of arterial blood flow may prevent cell death. Such cells are reversibly injured cell.

Reimer documented a wavefront phenomenon in which cell death occurs first in the subendocardium, then extends to the mid- and next to the subepicardium following coronary occlusion in dogs. Rona reported the presence of non-homogeneous cell injury in the ischemic

Key Words:
- Myocardial ischemia
- Ultrastructure
- PAS stain
- Reperfusion
- Ca**-activated adenosine triphosphatase

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cardiac muscle of the rat heart with coronary artery ligation, and found a correlation between milder ischemic injured cells and the presence of collaterals. These findings indicate that cells subjected to mild ischemia can recover by arterial blood reperfusion after a few hours of ischemia. Both beneficial and harmful effects on the ischemic and anoxic myocardium have been reported after revascularization and reoxygenation.\textsuperscript{5–15}

It is necessary to determine the limits within which revascularization can restore cardiac function. Since study of the isolated perfused heart and comparative studies among several groups of experimental models do not adequately resolve the issue of myocardial viability in vivo, further research is necessary on the process of ultrastructural changes caused by ischemia and subsequent recovery with reperfusion in the beating heart. In the present study we semi-quantitatively assessed the ultrastructural changes after ischemic injury followed by reflow in the beating heart, in order to clarify the extent and reversibility of ischemic injury during the early and late reperfusion phases. In addition, the recovery of Ca\textsuperscript{2+}-activated adenosine triphosphatase (Ca\textsuperscript{2+}-ATPase) activity in mitochondria (Mit) and sarcoplasmic reticulum (SR) was examined in the affected cells to evaluate reversibility.

METHODS AND MATERIALS

Thirty-three healthy adult mongrel dogs of both sexes, weighing 10 to 18 kg were anesthetized with sufficient intravenous sodium pentobarbital to abolish corneal reflex. Each dog was intubated with a cuffed endotracheal tube and ventilated with a mixture of room air and oxygen in order to maintain arterial blood oxygen partial pressure levels over 80 mmHg using an Aika R-60 respirator. During the experiment, six dogs died from ventricular fibrillation, and in another 5 dogs, adequate cyanosis was not achieved. The remaining 22 dogs were divided into two groups: (A) 8 dogs without reperfusion, (B) 14 dogs with reperfusion.

The chest was opened through the left fourth or fifth intercostal space. The left anterior descending artery (LAD) was isolated from the fatty tissue and a snare ligature was applied tightly below the first diagonal branch to obstruct the coronary artery for 60 minutes in both groups. In Group B the snare ligature was loosened for 2 hours to allow coronary reperfusion. The ligation produced a clearly and widely cyanotic area involving the apex region of acute ischemia corresponding to the distribution of the LAD distal to the occlusion. On reflow the cyanotic myocardium became pink within a period of seconds. A bolus dose of lidocaine (2 mg/kg) was administered intravenously as necessary to prevent premature ventricular contractions in 8 dogs. Lidocaine caused only a transient (5–10 min) decrease in heart rate and blood pressure.

The histochemical distribution of glycogen and ultrastructural alterations were examined in both groups. At the end of the experiment the hearts in Group A were rapidly excised and washed in ice-cold saline. Figure 1 shows the methods schematically.

A transmural myocardial block was cut, including the cyanotic edge, and specimens of approximately 1 mm\textsuperscript{3} were taken for ultrastructural study: (1) a mid-wall portion in the non-ischemic region, (2) a subepicardial portion and (3) a subendocardial portion in the center of ischemic region. Immediately thereafter, the remaining block was frozen in liquid nitrogen for histochemical glycogen staining. In Group B transmural myocardial needle biopsy specimens 5 mm apart were obtained for the ultrastructural study just before and after 5 minutes of reflow using a No.10 Silverman needle from sites in the central area of ischemia that were over 2 cm from cyanoletic border. At the completion of the experiment, a transmural myocardial block was cut as in Group A. A 120-minute reperfused specimen for ultrastructure was taken from the same three portions as in Group A. All specimens were immediately immersed in 2.5 percent glutaraldehyde in 0.1 molar phosphate buffer pH 7.4 for 3 hours. They were further fixed in buffered 1 percent osmium tetroxide for 3 hours, then dehydrated in graded ethanol series and embedded in Epon 812.

Ultrathin sections were cut with a DUPON MT2-B Ultra Microtome using a glass knife, and stained with uranyl acetate and lead citrate. Sections were observed on a HITACHI H-500 electron microscope.

For histochemical study, serial frozen sections (10 \textmu m) were cut at intervals of 20 \textmu m from a block and stained by the periodic-acid Schiff diastase method for glycogen.

Cytochemical detection of Ca\textsuperscript{2+}-ATPase was
performed by the method of Fujimoto and Ogawa. Cardiac muscles (biopsy specimens after 1 hour ischemia and 2 hours reflow) were cut into slices, less than 1 mm thick, and fixed in 2% paraformaldehyde in 0.1M cacodylate buffer, pH 7.4, for 30 min at 4°C. After fixation, tissues were washed in the same buffer with 0.25M sucrose for 60 min. Tissues were then cut on a Vibratome (Oxford, California) at 40 μm thickness. These sections were incubated in the following reaction medium for 15 min at 37°C: 250 mM glycine buffer, pH 9.0; 3 mM ATP-2Na (Sigma Chem. Co.); 10 mM CaCl₂; 4 mM lead citrate; 2.5 mM levamisol (Sigma Chem) as a non-specific alkaline phosphatase inhibitor. Incubated sections were then rinsed in 0.1M cacodylate buffer, pH 7.4, with 0.25M sucrose, and postfixed with 1% OsO₄ in 0.1M cacodylate buffer, pH 7.4, for 20 min. Serial dehydration in graded alcohol was followed by embedding in EPON 812. Ultrathin sections were prepared with a DUPON MT-2-B Ultra Microtome, and observed unstained under a HITACHI H-500 electron microscope.

Morphometric analysis of myocardial cell...
Reperfusion Effect on Ischemic Myocardial Injury

Fig. 3. A shows transmural infarction type after 60 minutes of coronary occlusion. There are many glycogen containing cells in the ischemic area. (PAS staining) B: Three-dimensional analysis revealed that the patchy distribution is peninsula of glycogen running parallel with the vessel.

Injury was performed on one of the ultrathin sections under electron microscopy. Each myocardial cell was observed in one to three myocardial cell layers along the edge of grid on which ultrathin sections were placed as shown in Fig. 2. The grid edge was placed over non-

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overlapping cells per section; therefore the same cell was never evaluated twice. Cytological alteration of the cells was graded according to criteria as shown in Table I. Almost seventy to one hundred cells in one section of each ultrathin section were assigned. The myocardial alteration score in each cell was graded from grade 0 (normal) to 4 (most severe). The mean alteration score was determined for both subendocardium and subepicardium by calculating the average severity (0–4) of myocardial cell injury. This scoring system has been useful in showing early reduction of ischemic damage by pharmacological intervention. The mean value indicates the degree of damage in the sample. The Student's paired t test was used for comparisons of pre-reperfusion (posts ischemia) vs. post-reperfusion states of both subepicardial and subendocardial damage, or subendocardial vs. subepicardial damage after 60 minutes of ischemia.

<table>
<thead>
<tr>
<th>TABLE I MYOCARDIAL ALTERATION SCORE</th>
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<tr>
<td>0 = normal myocardial cell</td>
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<tr>
<td>1 = less than 50% reduction in glycogen granules and vacuoles in the sarcoplasm.</td>
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<tr>
<td>minimal reduction in matrix density and slight swelling of mitochondria.</td>
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<tr>
<td>2 = a small number of glycogen granules in the sarcoplasm.</td>
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<tr>
<td>reduction of matrix density and partial disruption of cristae of mitochondria.</td>
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<tr>
<td>moderate intermyofibrillar edema.</td>
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<tr>
<td>3 = absence of glycogen granules in the sarcoplasm.</td>
</tr>
<tr>
<td>marked intermyofibrillar edema.</td>
</tr>
<tr>
<td>marked sarcolemmal lifting-off.</td>
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<tr>
<td>mitochondrial swelling with significant disruption or disappearance of cristae.</td>
</tr>
<tr>
<td>4 = severe swelling with significant disruption of myocardial cell.</td>
</tr>
<tr>
<td>amorphous dense body is present in the mitochondrial matrix.</td>
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</table>

RESULTS

Histochemical Study
All myocardial cells in non-ischemic area contained numerous glycogen granules observed under light microscopy; ischemia resulted in glycogen consumption in the sarcoplasm seen under electron microscopy. Therefore, we assessed the glycogen distribution as a marker for ischemia. In Group A, glycogen-depleted and -rich areas were sharply differentiated, although the glycogen distribution varied even after a 60-minute occlusion. A patchy distribution of glycogen was seen mainly in the subepicardial side, and areas with total cellular glycogen depletion intermingled with those which contained varying amounts of glycogen. Glycogen depletion on the subendocardial side was more marked and evenly distributed than on the subepicardial side. In some cases myocardial glycogen was present in the subepicardial half

Fig. 4. Glycogen distribution of group B (after 1-hour ischemia followed by a 2-hour reflow). Glycogen content is more numerous and widely distributed in the ischemic area.

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layer but absent or reduced in the subendocardial half layer (subendocardial infarction type). Most of the other cases showed the feature of transmural infarction. This is that myocardial glycogen was reduced in increasing severity from the epicardial to endocardial side with a small number of patchy areas of glycogen mainly in the subepicardial halves (Fig. 3-A).

Section to section tracing of these islands revealed peninsulas running with a vessel, and all islands could be traced back to glycogen-rich subepicardial layer. Reconstruction of infarction from these serial section, namely, three-dimensional analysis of the infarcts revealed no true islands but instead peninsulas running parallel with the vessel (Fig. 3-B).

In Group B, however, cells containing an intermediate glycogen content were more numerous and widely distributed than those in Group A, especially, on the subepicardial side. Glycogen content was more homogeneous in the subepicardium than in the subendocardium. There were numerous peninsulas in the glycogen-depleted area, suggesting recovery from ischemia by reflow (Fig. 4).

**Ultrastructural Study**

(1) After 60 minutes of LAD occlusion

The myocardium from non-ischemic sites in Group B appeared almost normal except for the presence of some vacuoles (Fig. 5). After ischemia, various degree of ultrastructural changes were observed such as nuclear chromatin clumping, wide I bands, reduction or loss of glycogen, intermyofibrillar edema, mitochondrial swelling with loss of matrix density and separation of cristae. In some severely damaged cells, the mitochondrial matrix was electron-lucent and most of the cristae disappeared with only remnants of the original structure left. An amorphous dense body was present in the
### Table II: Morphometric Analysis of Ischemic and Reperfused Myocardial Tissue

<table>
<thead>
<tr>
<th></th>
<th>Subepicardium</th>
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<th>Subendocardium</th>
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<tbody>
<tr>
<td></td>
<td>60 I</td>
<td>120 R</td>
<td>60 I</td>
<td>120 R</td>
</tr>
<tr>
<td></td>
<td>Degree of damage</td>
<td></td>
<td>Degree of damage</td>
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<tr>
<td>Group A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.20</td>
<td>0.24</td>
<td>1.13</td>
<td>1.86</td>
</tr>
<tr>
<td>2</td>
<td>1.13</td>
<td>*</td>
<td>2.44</td>
<td>*</td>
</tr>
<tr>
<td>3</td>
<td>0.15</td>
<td>0.75</td>
<td>0.19</td>
<td>0.11</td>
</tr>
<tr>
<td>4</td>
<td>0.81</td>
<td>3.47</td>
<td>0.27</td>
<td>0.56</td>
</tr>
<tr>
<td>5</td>
<td>1.95</td>
<td>*</td>
<td>3.39</td>
<td>*</td>
</tr>
<tr>
<td>Group B</td>
<td>9</td>
<td>1.30</td>
<td>0.91</td>
<td>3.10</td>
</tr>
<tr>
<td>10</td>
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<td>1.26</td>
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<td>1.39</td>
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<td>1.58</td>
<td>3.00</td>
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<td>18</td>
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<td>0.73</td>
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<td>19</td>
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<td>0.25</td>
<td>1.41</td>
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<tr>
<td>20</td>
<td>0.86</td>
<td>0.04</td>
<td>2.05</td>
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<tr>
<td>21</td>
<td>1.39</td>
<td>0.65</td>
<td>0.59</td>
<td>3.90</td>
</tr>
<tr>
<td>22</td>
<td>1.48</td>
<td>0.23</td>
<td>3.94</td>
<td>3.73</td>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>Group A + B</th>
<th></th>
<th>Group B</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>1.25 ± 0.71</td>
<td>*</td>
<td>1.99 ± 1.18†</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>1.46 ± 0.53</td>
<td>1.09 ± 0.65††</td>
<td>2.12 ± 0.96</td>
<td>2.15 ± 1.36</td>
</tr>
</tbody>
</table>

Numbers represent mean alteration scores.  
60 I = after 60 minutes of ischemia; 120 R = after 60 minutes of ischemia plus 120 minutes of reflow; † = Significant difference (p < 0.01) between subepicardium and subendocardium after 60 minutes of ischemia; †† = Significant difference (p < 0.05) between 60 I and 120 R of subepicardium. (mean ± S.D.)

An extensive range of ischemic damage was noted in the subepicardium, although it was also noted in the subendocardium (Fig. 6). The mean values of myocardial cells in the scoring system for each dog with or without reperfusion are presented in Table II. In ischemic myocardium, resultant ischemic change was essentially similar on both the subendocardial and subepicardial side, but the subendocardium showed a more significantly degree of damage (p < 0.01) than the subepicardium (Fig. 7-A, 7-D, Table II). This demonstrates the presence of a definite transmural gradient of the cellular damage. In Group B all cases had a mean alteration score of less than grade 3 in the subepicardium, although, 4 out of 14 cases were over grade 3 in the subendocardium.

(2) Early phase of reperfusion (Fig. 7-B, 7-E)  
A 5-minute reperfusion following 1 hour of ischemia resulted in dramatic changes in almost all of the cardiac muscle cells compared with those not receiving reperfusion. The most striking changes were massive swelling of both some of mitochondria and cell cytoplasm, numerous vacuolation, and hypercontraction of sarcomeres. Separation at the intercalated disc was occasionally observed. Some of mitochondrial matrix were electron-lucent and most
Fig. 7. A: Subepicardial cell after 60 minutes of occlusion. Cell shows alteration score of grade 1. Nuclear chromatin clumping, intermyofibrillar edema, some vacuoles and decreased glycogen granules are present in sarcoplasm. (x 10000)
B: Subepicardial cell with 60 minutes of ischemia plus 5 minutes of reflow. Massive swelling and numerous vacuoles are present with mild decrease in number of glycogen. (x 4800)
C: Subepicardial cell with 60 minutes plus 120 minutes of reflow. Cell shows alteration score of grade 1. There is no clumping of nuclear chromatin, numerous glycogen granules and normal matrix density of mitochondria. (x 7000)
D: Subendocardial cell after 60 minutes of ischemia. Cell shows alteration score of grade 3. Wide I band, nuclear chromatin clumping, intermyofibrillar edema, vacuoles and sarcolemmal membrane lifting off are seen. (x 6000)
E: Subendocardial cell with 60 minutes of ischemia plus 5 minutes of reflow. Cell shows severe contraction feature. Numerous contraction bands and vacuoles are seen. Mitochondria matrix is electron-lucent and most of cristae have disappeared leaving only remnant of the outer membrane. (x 7000)
F: Subendocardial cell with 60 minutes of ischemia plus 120 minutes of reflow. Cell shows alteration score of grade 2. Sarcomers units are regular without wide I band. Some mitochondria swelling with reduced matrix density and disruption of cristae are present. (×4800)

G: Subendocardial cell with 60 minutes of ischemia plus 120 minutes of reflow. There is a heterogeneity in the degree of recovery state in the vicinity. C1, C2 and C3 cells show alteration score of grade 2, 3 and 4, respectively. (×1800)

Samples of A, B and C, and of D, E, F and G were obtained from the same dog.

Mit = mitochondria; Nu = nucleus; Mf = myofibril; G = glycogen; V = vacuole; CB = contraction band

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Fig. 8. Cytochemical findings of Ca²⁺-ATPase.

(A) Ca²⁺-ATPase activity is localized at the mitochondria and sarcoplasmic reticulum (arrow) and myofilament (arrow head) in non-ischemic myocardial cell. (×12000, insert ×45000)

(B) A decrease in Ca²⁺-ATPase activity is recognized in a myocardial cell after 60 minutes of coronary occlusion. (×12000)

(C) A increase in Ca²⁺-ATPase activity is recognized in a significantly recovered myocardial cell with 120 minutes reflow. (×10000)

(D) Note no recovery of Ca-ATPase activity in severely damaged (over grade 3) myocardial cell. (×8000)

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of the cristae disappeared leaving only remnants of the outer membrane. Numerous vacuoles were present in the sarcoplasm, some of them appeared to originate from the mitochondrial outer membrane. Some of the ischemic cells exhibited numerous lipid droplets.

(3) Late phase of reperfusion

Three types of cellular formation were generally observed in myocardium receiving 120 minutes of reflow. The first type of myocardial cell (myocardial alteration score of grade 1, Fig. 7-C) resembled the control cells obtained from the non-ischemic sites except for a slight swelling of cytoplasm, a few vacuoles in the sarcoplasm and minimal reduction of matrix density in some of mitochondria. The second type of cell (myocardial alteration score of grade 2, Fig. 7-F) showed apparent recovery of the ultrastructural changes in both the subendocardium and subepicardium. This type of cell

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also contained apparent intermyofibrillar edema, a number of large and small intracellular vacuoles, but the wide I bands were no longer present in most of these cells. The nuclear chromatin no longer demonstrated margination or clumping. The new glycogen granules varied in number but were more numerous in the myocardium after reflow when compared to those at 60 minutes of ischemia only. In addition, numerous mitochondria with reappearance of the matrix density were seen. While some mitochondria showed separation of cristae even after this extended reperfusion, other mitochondria in the vicinity showed regular cristae and normal matrix density. Dilatation of both the sarcoplasmic reticulum and T-tubules were also present in the sarcoplasm in most of the cells. The third type of cell (myocardial alteration score of 3 and 4, Fig. 7-G) developed severe changes without signs of ultrastructural restoration. Severe disruption of the myofibrils with the loss of Z lines was noted. Some basement membrane was lost and the plasma membrane was extensively disrupted. Distortion and disorganization of the sarcomeres were common, and they were accompanied by numerous contraction bands that frequently appeared as a homogeneous gray mass from which myofibrils could scarcely be distinguished. Glycogen granules were absent in sarcoplasm in this type of cells. These cells contained large intracellular vacuoles and spaces, and the sarclemma was always markedly lifted off the Z band. In addition, there were occasionally amorphous dense bodies in the mitochondria. This type of cell showed marked clumping and the margination of nuclear chromatin and the nuclear outline was relatively blurred.

The use of semiquantitative techniques for grading ischemic damage facilitated characterization of the details of the ultrastructural appearance of cells both injured by ischemia and salvaged by reperfusion. The subepicardial cells showing myocardial alteration score below grade 3 clearly survived. They exhibited normalization of nuclear chromatin, reappearance of glycogen granules, presence of mitochondria with normal dense matrix and regular cristae, and normal figure of sarcomere units. After 2 hours of reperfusion, the subepicardium showed significant (p < 0.05) ultrastructural recovery (Table II). While in the subendocardium, no significant recovery was observed. The subendocardium showing severe injury (mean alteration score of greater than grade 3) at 60 minutes of occlusion exhibited little improvement (less than grade 2). In addition, reperfusion appeared to accelerate the development of the lesion in ischemic injured cells (Fig. 7-G). These findings demonstrate heterogeneity in the degree of damage following the occlusion, indicating that some cells had been reversibly damaged. Myocardial cells using biopsy technique as controls (without occlusion nor reperfusion) appear normal, indicating the biopsy procedure itself does not influence the ultrastructure of cells over 5 mm apart.

(4) Cytochemical study

Figure 8-A shows electron micrographs of the non-ischemic myocardial cell. Non-ischemic myocardial cell Ca**-ATPase reaction product was localized on the matrix of mitochondria (Mit), sarcoplasmic reticulum (SR), gap junction of intercalated disc and myofilaments (Mf). In the control experiment using Ca**-free medium, no reaction product was observed in any intracellular organelles or around the gap junctions. The intensity and loci of reaction product were stable and few structural changes were noted. Significant differences in the degree of the Ca**-ATPase activity were found between the 60 minute-ischemic and non-ischemic myocardium. The former showed marked reduction of the cytochemical reaction, especially in Mit, on SR and Mf (Fig. 8-B). However 120 minutes of reflow resulted in apparent recovery of Ca**-ATPase activity in cells with scores of less than grade 2, which were obtained from the center of a previously ischemic subendocardium (Fig. 8-C). In severely damaged cells, however, Ca**-ATPase activity in the organelles remained low (Fig. 8-D).

DISCUSSION

(1) The regional myocardial infarction induced by 1 hour coronary occlusion

In the past 10 years a number of approaches have been employed to reduce infarct size, such as reperfusion, intracoronary thrombolytic and surgical revascularization. Reimer et al speculated on the presence of a subepicardial zone of ischemic injury that contains some viable cells within 6 hours of ischemia. The rate of myocardial necrosis is known to differ widely in experimental animals from virtually no necrosis after several hours of coronary artery occlusion to transmural necrosis after only 40 minutes of ischemia, depending on the size of ischemic bed and the availability of collateral blood supply. Moreover, it has been reported.
that occlusion of the same coronary artery at the same anatomic site in a group of dogs resulted in significant variation in the size of the resultant infarcts.22

The present study analysed histochemically, ultrastructurally and cytochemically the changes induced by ischemia and the subsequent reflow using both cut blocks and biopsy specimens of the same beating heart. Histochemical findings of glycogen distribution after a 1 hour occlusion indicate that the subepicardium with more numerous glycogen-rich cells is less affected by ischemia than the subendocardium with glycogen-depleted cells. The phenomenon could be dependent on collateral flow from the findings of three-dimensional analysis. Our previous investigation using intracoronary dye injection suggested that the presence of peninsulas of glycogen-containing cells is related to collateral blood flow.23 It is noteworthy that ultrastructural features in ischemic damage over grade 2 revealed sparse or absent glycogen in the sarcoplasm. Thus glycogen-depleted cells included some of the ultrastructural ischemic damage of grade 2 cells and over grade 3 cells. Our data also revealed a transmural gradient of ischemic cell damage from the subepicardial to subendocardial side of the left ventricular wall. These findings suggest that less severely injured ischemic cells were present even in the subendocardium, and especially in subepicardium. Similar findings have been reported by Rona and Kloner et al.5,21,24,25 As cell death progresses at different rates in different regions of the heart, it is reasonable that while some of ischemic cells can be salvaged, others become necrotic after reperfusion.

(2) Reperfusion effect on ischemic myocardial cells

The present results of reperfusion following coronary artery occlusion appear to be inconsistent with histological and ultrastructural analysis, metabolic and pathophysiologic studies previously reported. Some authors5−9,26 have found that reperfusion developed intramyocardial hemorrhage, abnormal Q waves and ventricular arrhythmia, various destructive structural changes, serious hemodynamic abnormalities, abnormal rise of serum creatine phosphokinase and lactate levels, and abnormal changes of electrolytes in the heart. In view of these findings these authors concluded that reperfusion may be detrimental and may cause extension of necrosis.

On the other hand, early reperfusion has been shown to restore jeopardized ischemic myocardium.5,10,15 Constantini et al.8 demonstrated that prolonged reperfusion reduced mean infarction size and cardiac function. Although the myocardial cells have been extensively studied by electron microscopy under various conditions of temporary ischemia or anoxia followed by reflow or reoxygenation, most of the works was performed on isolated perfused hearts or involved comparative studies among several different groups of experimental animals.7,6,11−14 There are few reports describing the ultrastructural recovery process or irreversible process on reperfusion effects in transient ischemic myocardium in one beating heart.

The question is whether or not reperfusion injury occurs during the early phase of reflow. In biochemical studies on isolated perfused rat hearts subjected to transient anoxic reperfusion and then reoxygenation, the rate and amount of creatine phosphokinase release upon reoxygenation increased rapidly only in hearts with longer periods of anoxia13,27 Myocardial enzyme release occurs as a sudden catastrophic event when hearts injured by anoxia are reoxygenated (oxygen-induced enzyme release).14,15,18−21,24−27 or when calcium is resupplied to hearts following a period of calcium-free perfusion.28,29 According to Ganote et al.9 this enzyme release occurs within a very short time (10 minutes) when oxygen or Ca-ion are re-introduced to the hearts previously perfused with anoxic perfusion or Ca**-free medium respectively. Those investigators described that although different explanations can not be excluded, mechanical events associated with cellular contracture and the stretching and disruption of sarcotrama were potential explanations for enzyme release in both the calcium-paradox and oxygen-induced enzyme release. Increased leakage of enzymes induced by the reoxygenation could be due, to a large extent, to the disruption and displacement of mitochondrial cristae, and sometimes, the disrupted lining membrane of these organelles.12 Because membrane structures affected by anoxia no longer have sufficient energy stores to maintain morphological integrity, these membranes could easily be disrupted when oxygen was re-introduced.31 Whalen et al.26 noted that reperfusion of the previously ischemic myocardium resulted in abnormal changes of...
electrolytes. Several findings concerning contracted myofibrils in reperfused ischemic myocardium can be explained on the basis of elevated levels of intracellular calcium ion. It has also been suggested that intracellular calcium overload is involved in myocardial fiber necrosis, and it is likely that this is the mechanism of the observed extensive ultrastructural damage brought about by reperfusion of the ischemic myocardium. Sufficient evidence appears to be at hand to relate elevated cytosolic calcium levels, caused by modification of cell membrane permeability to this cation, to cell death. In the present study after 5 minutes of reperfusion each myocardial cell immediately developed some changes such as rapid swelling of both Mit and cytoplasm, abnormal contracture of sarcomeres, rupture of some of Mit leaving only remnants of the outer membrane and occasionally formation of amorphous dense bodies in the Mit in early reperfusion phase. These drastic changes were compatible with the phenomenon known as calcium paradox and oxygen paradox in respect to both ultrastructural changes and abnormal enzyme release. After 120 minutes of reflow, however, in cells with less than grade 3 ischemic damage many Mit showed minimal swelling and regular cristae. In addition, the wide I band was no longer present, and the nuclear chromatin no longer demonstrated margination or clumping. Although various numbers of glycogen granules were present in the sarcoplasm, they were undoubtedly more numerous than after ischemia. Interestingly, the cells showing recovery process and those showing necrosis existed side by side (Fig. 7-G). Viability of the affected myocardial cells was assessed cytochemically by a detection of Ca\(^{2+}\)-ATPase activity on in Mit, SR and Mf in this study. Disruption of intracellular homeostasis with respect to Ca\(^{2+}\) is an important factor in ischemia induced injury. It is generally accepted that the SR and Mit regulate the intracellular Ca\(^{2+}\) concentration by an active transport mechanism, with Ca\(^{2+}\)-ATPase in those organelles playing an important role. Ozawa reported that marked decrease in ATPase activity was considered to be one of the signs of ischemic irreversibility. Reperfused cells showing an ischemic grade of less than 3 maintain a high level of Ca\(^{2+}\)-ATPase activity in Mit and SR, indicating that these cells are in a viable state.

Schaper et al. described the ultrastructural criteria of irreversible ischemic injury which is characterized by mitochondrial clearing and large amorphous dense bodies within the Mit. In addition, disorganization or disruption of individual sarcomeric units are frequently present. The features of irreversibly injured cell defined by Schaper et al. were equivalent to ischemic grade 3 and 4 in the present study. Reversible ischemic injury was defined as inter-myofibrillar edema and swelling of Mit with loss of matrix density and partial disappearance of cristae, although amorphous dense bodies in Mit and marked sarcolemmal lifting-off have not developed yet. It appears that high grade ischemic injured cells are non-viable cells, because there is no evidence of recovery after 120 minutes of reflow. Compared with the subepicardium, there was a wide variance between cases showing good recovery and those not recovering, because the glycogen distribution in the subendocardium was not as homogenous as in the subepicardium. The various intensities of myocardial damage, not only after 60 minutes of ischemia but also after 120 minutes of reflow in the subendocardium, may depend on both the varying degrees of collateral circulation and the significantly reduced myocardial blood flow to the reperfused subendocardium compared to the flow to the non-ischemic and reperfused subepicardial region. However, Fujiwara et al. speculated that the transmural cell damage gradient may be the result of transmural gradient of wall stress and intramyocardial pressure using the pig heart lacking a collateral blood supply. Although the results of the present study suggest that the transmural gradient of collateral flow is correlated mainly with the rate of heterogeneous cell death, we can not exclude the possibility that transmural gradients of wall stress and intramyocardial pressure also play a role. Furthermore, another possibility with respect to the heterogeneity of ischemic damage in the ischemic area after 60 minutes of ischemia may be the difference in the tolerance of cells to ischemia, because an electron micrograph showed viable cell and non-viable cells to be in close vicinity as shown in Fig. 7-G. Burton et al. also described a mixed population of muscle cells including severely damaged cell and ultrastructurally normal cell in the vicinity in isolated feline myocardial cell subjected to hypoxia followed by reoxygenation.

Our results obtained using biopsy technique on the beating heart indicate the following items; (1) histochemical analysis by glycogen staining.
method revealed many peninsulas consisted of viable cells running parallel with the vessels in the ischemic area, (2) the degree of ischemic damage varied in intensity from slight to severe in each infarction case even in glycogen-depleted area, (3) a definite transmural gradient of cellular damage existed from the subendocardium to the subepicardium, (4) the ischemic cells, especially in the subepicardium, showing moderate injury at 60 minutes of occlusion made a significantly greater recovery in their ultrastructure after 120 minutes of reflow than those showing severe injury. This occurred although after 5 minutes of reflow, cells developed contraction bands, formation of vacuoles and swelling of mitochondria, and disruption of mitochondrial cristae. These observations were supported by the cytochemical findings in which temporally decreased Ca\(^{++}\)-ATPase activity after ischemia was returned to high activity level after reflow. (5) the presence of marked sarcosomal lifting-off and amorphous dense bodies in Mit of ischemic cells suggest irreversible injury.

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