Degrading Process of Acute Ischemic Myocardial Cells

TAKASHI KATAGIRI, M.D., EIICHI GESHI, M.D., TOSHIKUNI YANAGISHITA, M.D.
NOBURU KONNO, M.D., AND HIROFUMI TANNO, M.D.

1. Development of acute ischemic myocardial injury was studied in mongrel dogs, induced by ligation of left anterior descending coronary artery (LAD), by biochemical analysis of myocardial fractions such as sarcoplasmic reticulum (SR) and mitochondria (Mt) and by electron microscopic observation of ischemic myocardial cells with lanthanum probe method.
2. Irreversible injury of ischemic myocardium initiated in subendocardial muscle as early as 20 min after occlusion of LAD as expressed degradation of major ATPase protein and phosphatidylcholine and phosphatidylethanolamine of SR and irreversible impairment of state III respiratory and dinitrophenol (DNP)-ATPase activities of Mt, and these necrotic changes advanced to subepicardial layer at about 60 min.
3. Ultrastructural irreversible findings appeared later at about 60 min following inflow of lanthanum ions in ischemia for 30 min.
4. Activation of cathepsin B inside of SR under ischemic acidic metabolism and abnormal inflow of Ca^{++} into ischemic cardiac myocytes are suggestive of very important factors for the initiation of myocardial ischemic injury in early myocardial ischemia.

NOWADAYS many clinical efforts have been attempted to reduce the size of myocardial necrosis by the reconstruction of the coronary blood flow employing such interventions as intracoronary thrombolysis (ICT) with urokinase (streptokinase in USA) or tissue plasminogen activators and percutaneous transluminal coronary angioplasty (PTCA) in the early stage of acute myocardial infarction.¹ As to the availability of these measures to clinical practice, the absolute viability of the ischemic myocardium should be inevitable for the salvation. The acute ischemic injury has been described to initiate in severe ischemia as shortly as 20 to 30 min in dogs²; however, in those experimental

works the irreversibility of the ischemic heart muscle was described to be 60 min to several hours later even with electron microscopy.³ In clinical practice, an anginal attack for longer than 20 to 30 min has been in common sense believed to produce more or less myocardial irreversible injury. In this series of studies we have investigated the time course of the development of ischemic irreversible injury and tissue reactions which lead to irreversibility under severe ischemia.

MATERIALS AND METHODS

1. Animal experiments: Total 180 adult mongrel dogs, weighing 10 to 24 kg, were anesthetized with a single intravenous injection of sodium pentobarbital at 35 mg/kg body weight, intubated and ventilated with room air with a Harvard type respirator. The left anterior

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The Third Department of Internal Medicine, Showa University School of Medicine, Tokyo, Japan

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Mailing address: Takashi Katagiri, M.D., The Third Department of Internal Medicine, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142, Japan

Fig.1. Electron micrographs of ischemic subendocardial cells reacted with lanthanum chloride.

A: non-ischemic cardiac myocyte (class-I cell), B: 30 min of ischemia (class-II cell), and C: 60 min (class-III cell). Lanthanum was localized only in the extracellular space in the non-ischemic myocardial cell, but was deposited inside in a cell in ischemia for 30 min without marked ischemic fine structural changes. In C, mitochondrial dense deposits were recognized with marked deposition of lanthanum. Bars indicate 1 μ m.

descending coronary artery (LAD) was totally ligated with a silk thread just below the first diagonal branch. Production of severe transmural ischemia of the anterior wall was checked by ischemic ST segment elevation of greater than 0.5 mV in the leads near V_3 and V_4 in electrocardiography. Five to 6 dogs were sacrificed at 15, 20, 30, 60, 120, and 180 min after LAD ligation. About 5 grams of the post-ischemic cyanotic heart muscle of the anterior left ventricular wall including ventricular septum was excised as a sample specimen simultaneously with the posterior portion of the left ventricle perfused by the circumflex branch as a non-ischemic control. Both of the ischemic and non-ischemic heart muscles were separated at the middle of the muscle layer and regarded as subendocardial (Endo) and subepicardial (Epi) muscles.

2. Lanthanum probe method: Heart muscles of Endo were cut into 1 mm³ mass with scissors and incubated in a reaction media containing 4 mM LaCl_3 and 0.1 M Na cacodylate (pH 7.4) at room temperature for 10 min, and rinsed 3 times in a solution containing 2% glutaraldehyde and 0.1 M Na cacodylate (pH 7.4).⁴ The

specimens were postfixated with 1% OsO_4 , and embedded in Epon 812 after dehydrated on a series of ethanols. They were cut into 0.1 μ m thick slices with a Sorvall MT-2 ultramicrotome, and observed with a Hitachi H-300 electron microscope with or without double staining with uranylacetate and lead nitrate. The severity of ischemic injury of cardiac myocytes were graded into 3 classes according to the report of Ozawa et al⁵ and compared with the deposition of lanthanum. Namely, they are class-I cells; normal or with very slight changes in which fine structures were preserved almost intact, class-II cells; cardiac myocytes with moderate changes, in which such changes as depletion of glycogen, accumulation of chromatin to the margin of the nuclear envelope, and slight mitochondrial swelling which have been reported likely to be the reversible changes, and class-III cells; myocardial cells with severe intracellular edema, dysarrangement and disruption of myofibrils and the appearance of mitochondrial dense deposits which have been regarded to be irreversible changes.

3. Fractionations of heart muscle: Heart

Fig.2. Distribution of ischemic injury of cardiac myocytes and lanthanum deposition. Quoted from Tanno et al⁴ with authors' permission.

Fig.3. Ca^{2+} -dependent ATPase activity of SR from ischemic myocardium. Quoted from Konno et al⁶ with authors' permission.

muscle specimens were minced with scissors, homogenized with a Polytron homogenizer and separated to mitochondria (Mt), sarcoplasmic reticulum (SR), lysosome rich, and cytosolic fractions by centrifugation procedure^{6,7} and the activities of the specific enzymes were determined as described in the previous articles. SR was prepared as the microsome fraction of heart

muscle, and Ca^{2+} -stimulated ATPase activity was measured. And protein fractions were analysed by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS)⁶. Mt was prepared utilizing alkaline protease and state III respiratory activity was determined by polarographic method utilizing succinate as the substrate. Activities of complexes I, II, and V

Fig.4. SDS gel electrophoresis of SR composing proteins. Note the reduction in the band of major ATPase protein in the ischemic myocardium. Endo: subendocardial, and Epi: subepicardial muscles. Quoted from Konno et al,⁶ with authors' permission.

Fig.5. State III respiratory activity of mitochondria from the ischemic heart muscle. Quoted from Geshi et al,⁷ with authors' permission.

(dinitrophenol-dependent ATPase) were measured as described elsewhere.⁷ Membrane composing phospholipids of SR and Mt were fractionated by 2 dimensional thin layer chromatography and content of each phospholipid was determined by the measurement of inorganic phosphate.⁸

RESULTS

1. Ultrastructural changes in the ischemic myocardial cells and deposition of lanthanum: Fig. 1-A-C shows electron micrographs of non-

ischemic and ischemic cells in Endo with La^{+++} probe. As shown in Fig. 1-A, in a class-I cardiac myocyte, lanthanum was deposited only in the extracellular space, i.e. on the glycocalyx of the cell wall and in the intercalated disc. Lanthanum was also observed in T-tubules, however, it was not found at all inside of the myocyte.

In ischemia for 30 min, lanthanum deposition was recognized in about 20% of the ischemic subendocardial cells disregarding relative preservation of fine structures and the absence of irreversible changes as presented in Fig. 1-B. At 60 min after coronary occlusion, the findings of

Fig. 6. Dinitrophenol-stimulated ATPase activity of the submitochondrial particles from the ischemic myocardium.

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ischemic cellular necrosis became apparent, such as severe intracellular edema, derangement of myofibrils, and swelling and partial breakdown of mitochondrial cristae. And so-called intra-mitochondrial dense bodies were found in swollen mitochondria. Lanthanum deposit was observed plentifully in the ischemic myocardial cells, as shown in Fig. 1-C, mainly in the cytosolic space and on the myofibrils. Sometimes lanthanum was found in the injured mitochondria. Fig. 2 presents the relation of the distribution of the ischemic myocellular injury and the deposition of lanthanum.⁴ In the class-I myocardial cells the fine structures were well preserved and the deposition of lanthanum was not observed at all inside of the cell. As early as 30 min after coronary ligation, lanthanum deposit was already found in about 20% of the ischemic myocytes in which irreversible fine structural changes were not apparent. In ischemia for 60 min, the numbers of cells with lanthanum deposition increased to 30% and the class-III cells occupied about 26% of the cells submitted to this study. And at 180 min of ischemia, greater than two-thirds of cells belonged to class-III in the ischemic Endo zone and lanthanum was deposited in most of the necrotic cells.

2. Degradation of SR: Fig. 3 shows Ca^{++} -stimulated ATPase activity of SR in the ischemic

myocardium.⁶ In the non-ischemic heart muscle, Ca^{++} -ATPase activity was always higher in Epi than that in Endo. In the ischemic myocardium Ca^{++} -ATPase activity decreased significantly in Endo as early as 20 min after coronary ligation. The activity decreased further to about 35% of the non-ischemic myocardium, but the further decrease was not obtained. In Epi, Ca^{++} -ATPase activity decreased later, i.e. at 30 to 60 min after coronary occlusion. And the activity in Epi was higher than that in Endo until at 180 min after coronary occlusion.

Figure 4 indicates electrophoretic gels of SR. As shown in the previous article,⁶ SR composing proteins were separated in the order of the molecular size, into the major ATPase, several bands of acidic proteins, and proteolipids. The major ATPase protein occupied about 40% of total SR proteins. In Endo, the band of the major ATPase decreased significantly at 30 min after coronary occlusion, and in Epi at 60 min, with relative increases in another proteins. These decreases in the major ATPase were roughly in parallel with the reduction in Ca^{++} -ATPase activity.⁶

3. Impairment of mitochondrial respiration: Fig. 5 shows state III respiratory activity of Mt from the ischemic heart muscle. The activity reduced significantly as early as 20 to 30 min

Fig.7. Fractionation of membrane composing phospholipids of SR from the ischemic myocardium.

Total contents of phospholipids were expressed as the amount of inorganic phosphorus on the left open bars.

Quoted from Yanagishita et al.⁸ with authors' permission.

Fig.8. Chronologic illustration of ischemic degradation of the microorgans of the heart muscle.

after coronary ligation in Endo, and at 60 min in Epi. State IV respiratory activity did not alter in ischemia for upto 120 min, therefore, so-called respiratory control ratio (RCR), ranged 6.03 ± 0.25 in Endo of the non-ischemic heart muscle, underwent in the similar manner as the

activity of state III.

Figure 6 shows dinitrophenol (DNP)-dependent ATPase activity of Mt from the ischemic myocardium. DNP-ATPase activity decreased significantly in Endo in ischemia for 20 to 30 min followed by the reduction in Epi at 60 min

Fig.9. Schematic illustration of ischemic myocardial necrosis.

after coronary occlusion. NADH-CoQ reductase (complex-I) activity also reduced in Endo in ischemia for 20 to 30 min in parallel with DNP-ATPase activity (data not shown), and these reductions in the activities corresponded well in chronology to the decrease in state III respiratory activity.⁷

4. Degradation of membrane composing phospholipids: Phospholipid contents of SR were presented in Fig. 7. The pattern and composition of phospholipids were very similar both in SR and Mt, as described elsewhere.⁸ In SR, total phospholipid content decreased significantly in ischemia from 30 min, as shown by open bars in the left column of Fig. 7. And phosphatidylcholine and phosphatidylethanolamine, the major components of SR composing phospholipids reduced mainly. Mt-composing phospholipids followed the similar manner as those of SR though they are not shown.

DISCUSSION

The length of ischemic period during which myocardial necrosis is generated has been recognized to be 20 to 30 min in many experimental and clinical studies.^{2,3} In experimental works utilizing ligation of the coronary artery in the dog heart, cease of coronary blood flow for 18 to 22 min was reported to be the critical time for the initiation of necrosis in Endo, and myocardial necrosis spreads from Endo to Epi with the lapse of time like a "wave-front phenomenon".⁹ In the clinical practice, it has been

believed to be one of the common senses that an anginal attack for longer than 20 to 30 min leaves myocardial necrosis more or less behind. However, the necrotic findings of the ischemic cardiac myocytes become recognizable after 40 to 60 min even with the observation by an electron microscope, and the ischemic myocardium remains like as reversible for several 10 min before the appearance of irreversible findings.

In this paper the results of our study group were summarized which have been performed to elucidate the process of myocardial necrosis in the early ischemic period when morphologic myocardial alterations of necrosis could not be detected even with electron microscopy.

As shown in this paper, the initiation of myocardial ischemic necrotic changes was detected in the membranous microorganelles of cardiac myocytes. They are degradations of the major ATPase protein⁶ and the major components of membrane composing phospholipids, phosphatidylcholine and phosphatidylethanolamine,⁸ of the microsome fraction which we regarded to be mainly SR, and irreversible reduction in mitochondrial aerobic respiratory function. Degradation of the major ATPase protein is particularly recognized quantitatively as the grade of necrotic changes advanced.⁶

Mitochondrial respiratory activity also impaired irreversibly in the early ischemic myocardium.⁸ The reduction in state III activity, an index of mitochondrial function to synthesize ATP under aerobic metabolism, advanced in the similar grade to the degradation of the major

ATPase protein and of phospholipids of SR in ischemia for 20 to 30 min at that time only slight ultrastructural alterations were recognized in electron microscopy.⁴ From these facts, it is evident that degradations of membrane structures of such microorganelles as SR and Mt occurred in the very early ischemia, and these are conceivable of the initiation of irreversible, i.e. necrotic, changes, because the destruction of membrane of microorgans such as SR, sarcolemma and Mt indicates the loss of not only function but also basic structures of the ischemic myocytes.

With the induction of ischemic myocardial necrosis many mechanisms have been proposed. In this report 2 mechanisms were described to be very likely. One is the activation of acid hydrolases inside of SR in the acidic state under the ischemic anaerobic metabolism.^{6,10} Although more than 20 of acid hydrolases was believed to be condensed in lysosomes, nowadays they were also found to be localized in SR.^{11,12} Among these hydrolases Konno and the co-workers⁶ elucidated the activation of cathepsin B in the acidic state was very likely to play a very important role in the initiation of degradation of the major ATPase of SR. Namely, cathepsin B is maximally activated in inside of SR, at a pH of 6.5 to 6.0 in the anaerobic acidic condition, and digest SR composing proteins, especially the major ATPase, from inside.¹¹ Another is the excess inflow of Ca^{++} from outside of the sarcolemma. This was proved by the deposition of lanthanum in the ischemic cardiac myocytes before the appearance of ultrastructural changes. Because La^{+++} has similar ionic diameter to Ca^{++} , and has been used as a Ca^{++} antagonistic substance,¹³ the deposition of La^{+++} would likely be to the proof of excess Ca^{++} inflow.¹⁴ And abnormal reactions which would be generated by the excess Ca^{++} such as activations of phospholipases A and C also digested the membrane structures in the ischemic myocytes. According to the thin layer chromatographic pattern of microsomal phospholipids from the ischemic myocardium, activation of phospholipase C was more likely to be responsible for the degradation of the ischemic cardiac myocytes.⁸

In conclusion of this article, the summary of the results of our series of studies and the proposed scheme of degradation of the ischemic myocytes are presented in Figs. 8 and 9, respectively. From these results, the ischemic degrading reaction advances very rapidly in Endo within

20 to 30 min of ischemia, and spreads to Epi.

Nowadays the attempts to reconstruct coronary blood flow are in fashion in clinical practice by the selective intracoronary administration of fibrinolytic enzymes such as urokinase and streptokinase, or tissue plasminogen activator, and by PTCA. These measures are likely to reduce the infarct size if they will be available in the very early stage of unstable angina pectoris or acute myocardial infarction, however, it would be very difficult to inhibit the initiation of necrotic reaction in Endo because of the limited time. And the application of new cardio-protective measures will be expected for the aid of salvage of the ischemic myocardium.

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