MORPHOLOGIC AND BIOCHEMICAL STUDIES ON THE EXPERIMENTAL CHRONIC ISCHEMIC MYOCARDIUM WITH THE AMEROID CONSTRICTOR*

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1. Morphologic as well as biochemical alterations in chronic ischemic myocardial tissue without infarction were studied in dogs utilizing the Ameroid constrictor.
2. Serum creatine kinase** activity elevated at around three weeks after placing the Ameroid constrictor around the circumflex branch of the left coronary artery suggestive of myocardial tissue injury followed by the initial activation caused by surgery.
3. Subendocardial proliferation of connective tissue was observed in about 60% of the experiments, but the middle and the subepicardial muscles were morphologically intact.
4. The marked increase in glycogen particles was observed in the subendocardial muscle cells in most of the experiments, and mild features of myocardial cellular necrosis were found in approximately 60% of the experiments.
5. ATPase activities of the structural proteins as well as sarcoplasmic reticulum in the ischemic myocardium showed relatively higher values than those in the non-ischemic myocardium. However, no substructural changes were observed in SDS gel electrophoresis in both the fractions.
6. The alterations in the chronic myocardial ischemia are supposed to be essentially the same as those in myocardial necrosis followed by acute coronary occlusion.

CHRONIC ischemic heart diseases, such as coronary sclerosis, coronary insufficiency, angina pectoris, etc., which are ordinarily the

preceding states of myocardial infarction, usually reveal fewer positive findings in clinical practice, such as ST-T changes in electrocardiogram and increased lactate content in coronary venous blood. And the morphologic as well as functional changes of the myocardium itself in the chronic ischemic state have not sufficiently been elucidated yet. This is because of the methodological difficulty to make chronic myocardial ischemia experimentally. In this paper chronic ischemic

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** Abbreviations were used in this article as follows: S-CK, serum creatine kinase; ATPase, adenosinetriphosphatase.

myocardium without massive necrosis was produced by placing an Ameroid constrictor around the coronary artery in the dog, and the fine structural and biochemical properties of the heart tissue in chronic ischemia were studied.

MATERIALS AND METHODS

All the chemicals were the reagent grade. Ameroid constrictors were obtained from Three Point Co. in Canada.

Animal experiments; Twelve adult mongrel dogs, weighing 10 to 15 kg, were anesthetized with intravenous administration of sodium pentobarbital (Nembutal®) at 30 mg per kg body weight. Under a regulatory respiration of room air through tracheal intubation, thoracotomy was performed at the left 4th intercostal space. The circumflex branch of the left coronary artery was isolated out of fatty tissue near of the ramification for about 5 mm long, and an Ameroid constrictor of the adequate size of the inner diameter with a metal sleeve was placed around the circumflex branch. The chest was closed and the dog was fed until gradual occlusion was produced in the coronary artery by the swelling of Ameroid plastic substance. In 8 of 12 dogs blood samples were obtained in each two to three days to check S-CK activities. At 4 to 5 weeks after operation the dogs were sacrificed under an anesthesia with Nembutal®. The beating heart was removed immediately and the posterior part of the left ventricular wall including the posterior papillary muscle was obtained according to the circulating area of the circumflex artery as well as the anterior portion of the left ventricle as a control. For electron microscopic study the central portion of the ischemic left ventricular tissue was divided into 3 parts, i. e., the subendocardial, the middle and the subepicardial portions, and fixed immediately with glutaraldehyde and osmium tetroxide. The remaining tissue was packed in ice for biochemical preparations.

Measurement of serum creatine kinase activity; S-CK was assayed by the method of Okinaka and the co-workers¹ with addition of cysteine as a stimulator. The transphosphorylated phosphate was assayed by the method of Fiske and SubbaRow²

Electron microscopic study; Ordinary glutaraldehyde-osmium tetroxide double fixing was performed. The myocardial tissue was cut into small blocks with a razor blade in a solution containing 2% glutaraldehyde, 5% sucrose and 0.1 M

sodium cacodylate (pH 7.4) and kept for 1 hour. Then the blocks were immersed in 1% osmium tetroxide and 0.1 M sodium cacodylate solution (pH 7.4) for another 1 hour. The fixed sample was embedded in Epon 812 and was sliced with a glass knife on a Porter-Blum ultramicrotome, and observed with a Hitachi-HS-9 electron microscope.

Preparation of sarcoplasmic reticulum; Preparation of sarcoplasmic reticulum was performed principally by the method of Harigaya and Schwartz.³ Polytron was used for homogenization and 5 mM sodium azide was added in homogenization and preparation to minimize mitochondrial activity. Hitachi 65-P ultracentrifuge and polyethylene tubes were used in precipitation of the microsome fraction. Protein concentration was determined by the biuret procedures⁴

Preparation of structural proteins; The crude fraction of myocardial tissues containing the total structural proteins were prepared by the method described in the previous paper⁵ The precipitate of the first 9,000 X G centrifugation in the preparation of sarcoplasmic reticulum was suspended in 9 volumes of 0.1 M NaCl and 0.01 M NaHCO₃ solution and the crude structural proteins were extracted. The last precipitate was divided into two parts and prepared for SDS gel electrophoresis and for ATPase assay as the manner described in the previous paper⁵

SDS polyacrylamide gel electrophoresis; SDS polyacrylamide gel electrophoresis was performed principally by the method of Weber and Osborn.⁶ 5 and 10% acrylamide gels were used in each sample with 4.1 and 2.7% of methylene-bis-acrylamide with respect to acrylamide, respectively. Electrophoresis was performed at a constant current of 8 mA per gel approximately for 3 and 4 hours in 5 and 10% acrylamide gels, respectively.

Measurement of ATPase activities; ATPase activities of sarcoplasmic reticulum and the total structural proteins were examined by the modified methods of Mead and the co-workers⁷ and Sugita and the associates,⁸ respectively. Reactions were performed in the reaction media as follows: 0.02 M tris-maleate (pH 7.2), 0.1 M KCl, 0.01 M MgCl₂, 10⁻⁵ M CaCl₂, and 0.005 M NaN₃ for sarcoplasmic reticular ATPase assay, 0.02 M tris-maleate (pH 6.8), 0.03 M KCl, 2 X 10⁻⁵ M CaCl₂, 0.001 M MgCl₂ and 0.005 M NaN₃ for actomyosin-ATPase assay, and 0.02 M tris-maleate (pH 6.5), 0.6 M KCl, 0.01 M CaCl₂ and 0.005 M

Fig.1. Chronological changes of serum creatine kinase activities. The second elevation of the activities at around three weeks suggests myocardial damage by the Ameroid constrictor.

NaN_3 for myosin-ATPase assay, in 0.005 M ATP at 25°C . 200 micrograms of the sample was applied in a total volume of 1 ml. The amounts of phosphate liberated in the reactions were assayed by the method of Fiske and SubbaRow.²

Measurement of Ca^{++} binding activities; Ca^{++} binding and Ca^{++} uptake were examined by the modified method of Harigaya and Schwartz,³ as reported by Toba and the co-workers.⁹ Millipore filter method was employed and 0.01 microcuries/ml of ^{45}Ca was mixed in the reaction media. 200 and 50 micrograms of sarcoplasmic reticulum in a total volume of 1 ml were used for examination of Ca^{++} binding and Ca^{++} uptake, respectively. The reactions were performed in the final concentrations of Ca^{++} of 10 μmoles and 100 μmoles , for 60 seconds and 30 minutes, for Ca^{++} binding and Ca^{++} uptake, respectively. ^{45}Ca taken by sarcoplasmic reticulum was counted in PPO, POPOP and Triton X-100 solution with an Aloka LSC 602 liquid scintillation counter.

RESULTS

Figure 1 shows S-CK activities after the operation of the Ameroid constrictor attachment. The initial rise of activities, which was observed on the next day to the following day after oper-

ation, was assumed to be caused by skeletal muscle injury in the surgery. S-CK activity elevated again at around three weeks after operation. This second rise of activity occurred in 5 of 8 dogs in which S-CK activities were examined, and was assumed to come from the myocardial injury caused by occlusion of the coronary artery by swelling of Ameroid plastic. The pattern of the second increase in S-CK activities was not the same in each dog. As shown in Figure 1, in some case the activities kept their high level constantly, but in another dog the raise was mild and temporary. However, in 3 dogs, the second rise of activities could not be observed. The difference of the changing pattern in S-CK activities was thought to be derived from the different mode and extent of development of collateral circulation.

All of the Ameroid constrictors, placed around the coronary artery, showed more than 50% of reduction in their lumen diameters, i. e., greater than 75% of decrease in the cross-sectional areas, at the sacrifice at 24 to 35 days after operation. Those were corresponding to the description of Vineberg and the co-workers.¹⁰ In one out of 12 dogs, a massive myocardial infarction was observed in the posterior portion of the left

Fig.2. Histological findings of the subendocardial layer of the posterior wall of the left ventricle. Proliferation of connective tissue is found at the left upper portion.

(H-E, $\times 100$)

Fig.3. Electron microscopic findings of a cardiac muscle cell in the subendocardial layer. A large conglomeration of glycogen particles is found. A bar indicates 1.0 micron.

Fig.4. Electron microscopic findings of a cardiac muscle cell in the subendocardial layer. Dense bodies are found in mitochondria. The cristae are vesicular formed. A bar indicates 1.0 micron.

Fig.5. 5 and 10% SDS acrylamide gels showing the structural proteins, the supernatant of 9,000 x G centrifugation and isolated sarcoplasmic reticulum.

Fig. 6. Ca^{2+} - Mg^{2+} -ATPase activities of sarcoplasmic reticulum of the myocardial tissue of the left ventricle. ○; non-ischemic portion, ●; ischemic portion.

Fig. 7. ATPase activities of the structural proteins of the myocardial tissue of the left ventricle. ○; non-ischemic portion, ●; ischemic portion.

Fig. 8. Ca^{2+} binding activities of sarcoplasmic reticulum of the myocardial tissue of the left ventricle. ○; non-ischemic portion, ●; ischemic portion.

ventricle including the posterior papillary muscle, and data were excluded in this paper. Another 11 hearts were almost normal in their appearances.

In the light microscopic studies, however, proliferation of connective tissue was observed in the subendocardial layer. This subendocardial fibrosis was observed in 7 of the 11 cases. One of these is shown in Figure 2.

Figures 3 and 4 show the electron microscopic findings of the subendocardial muscle cells in the ischemic left ventricle. As shown in Figure 3, a large conglomeration of glycogen particles were observed. However, the fine structures of the microorganellae, the arrangement of myofilaments, mitochondria, etc., were relatively kept intact. This increase in glycogen particles was observed in almost of the experiments. In the cases in which subendocardial fibrosis was observed, the features of cellular necrosis were seen, i.e., dense bodies, swelling and disruption of the cristae and decrease in the density of the substrate, in mitochondria. In these muscle cells, sarcolemma, the arrangement of myofilaments, sarcoplasmic reticulum, however, were not so altered in their appearances. Those changes in the fine structures were much slighter compared

to those of acute total occlusion of the coronary artery.¹¹ Also, the fine structural changes were limited to the cells in the subendocardial layer, and cells in the middle and the subepicardial layers were structurally normal.

Figure 5 shows the gel electrophoretic patterns of the myocardial fractions, i.e., structural proteins, the supernatant of centrifugation at 9,000 XG which includes sarcoplasmic reticulum and cytoplasm, and the isolated sarcoplasmic reticulum. In the structural proteins of the non-ischemic heart muscle, the patterns, mobilities, i.e., the molecular weights, and the compositions of the protein bands were the same as those in the previous report.⁵ There found no differences between in the non-ischemic and in the ischemic portion. In the 9,000 XG supernatant and the isolated sarcoplasmic reticulum, the patterns and the mobilities of the bands were almost the same in the non-ischemic and ischemic myocardium.

Ca^{++} - Mg^{++} -activated ATPase activities of sarcoplasmic reticulum were shown in Figure 6. Ca^{++} - Mg^{++} -stimulated ATPase activity was 0.041 $\mu\text{moles Pi/mg/minute}$ in the non-ischemic portion of the myocardium on the average. In the ischemic heart muscle, however, the slightly elevated activity of 0.05 μmoles was obtained. The difference of the activities was statistically significant ($P < 0.05$). However, basal Mg^{++} -activated ATPase activities of sarcoplasmic reticulum were equal in the non-ischemic and ischemic heart muscle.

Actomyosin- and myosin-ATPase activities of the structural proteins were shown in Figure 7. Actomyosin-ATPase activity was 0.105 $\mu\text{moles/mg/minute}$ and 0.120 μmoles in the non-ischemic and ischemic portion, respectively, and the difference of the activities was statistically significant ($P < 0.05$). Myosin-ATPase activity, however, was 0.117 $\mu\text{moles Pi/mg/minute}$ in the non-ischemic portion and 0.115 μmoles in the ischemic portion on the average, respectively, and the difference was statistically insignificant.

Figure 8 shows Ca^{++} binding and Ca^{++} uptake of sarcoplasmic reticulum. In three experiments the same values were obtained in sarcoplasmic reticulum from the non-ischemic and ischemic myocardium.

DISCUSSION

The morphologic and functional alterations of the chronic ischemic myocardial tissue, which would ordinarily be the preceding appearance of myocardial infarction, have been an interesting

subject for a long time. In this experimental study, a method using the Ameroid constrictor, which was designed by Vineberg and the co-workers,¹⁰ were introduced to make a chronic ischemic heart tissue. Production of chronic myocardial ischemia without massive necrosis in the left ventricle was expected by placing an Ameroid constrictor around the left circumflex coronary artery, with which the myocardial tissue of the circulating area by the corresponding vessel would have become ischemic by the gradual occlusion of the constrictor but would not have become massively necrotic with the development of collateral circulation from the anterior descending branch and the right coronary artery.

The difference in the level of S-CK activities, morphologic and biochemical changes in each individual dog suggests the different development of collateral circulation as well as the different type of coronary artery distribution.

The subendocardial proliferation of connective tissue was so mild that the change could not be detected as subendocardial infarction, however, the fibrosis was observed in most of the experiments in which S-CK activities elevated, and was thought to be the results of ischemic myocardial change. In the subendocardial cells the findings of mild to moderate features of cellular necrosis were found. In the near-subendocardial cells, dense bodies in mitochondria were observed with relative preservation of the finer structures of another organellae. These ultrastructural findings indicate that the light microscopic changes were of the ischemic type. Jennings and the associates¹² reported that the subendocardial layer would be most greatly influenced by the diminished coronary flow. The histological as well as electron microscopic changes in the subendocardial layer would correspond to their observations. The increase in glycogen particles in the subendocardial muscle cells was observed in most of the experiments, and this finding was the most characteristic in the chronic ischemic myocardial cells. The cause of this increase is still not established, however, the same phenomena were reported in hypothyroid myocardial cells.¹³ The increase in glycogen particles is conceived as one of the mode of the lowered cellular metabolism.

In comparison with the morphologic alterations, biochemical studies showed relatively poor results. The mild increase in ATPase activities of the structural proteins and sarcoplasmic re-

ticulum were still undissolved. The author reported the increase in actomyosin-ATPase activities in the initial stage of myocardial infarction.⁵ This would be the same phenomenon with the chronic ischemic heart tissue. Kashiwakura and the co-workers¹⁴ reported activation of nucleic acid metabolism in the experimental myocardial infarction. Under such circumstances as the initial phase of cellular degeneration activation of some enzymic activities might take place. However, neither any substructural changes were found in the structural proteins as well as sarcoplasmic reticulum, nor Ca^{++} binding and Ca^{++} uptake of the sarcoplasmic reticulum. Lee and the associates¹⁵ described reduced Ca^{++} uptake of sarcoplasmic reticulum in 90 minutes of ischemia. Toba and the co-workers⁹ recognized one of the major protein bands of microsome fraction decreased at 3 hours in the similar experiment. The absence of these substructural and functional changes in the chronic ischemic myocardium would indicate that the intensity of ischemia was much slighter than in acute occlusion of the coronary artery, and that the ischemic damage was limited to the subendocardial restricted portion.

However, as shown in the electron microscopic observations, the essentials of the changes in chronic ischemic myocardial cells are the same as those in the early phase of myocardial infarction.

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