STUDIES ON COLLAGEN IN THE EXPERIMENTAL MYOCARDIAL INFARCTION

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The healing process of myocardial infarction was studied with special reference to the production of collagen by the determinations of prolylhydroxylase (PHase) activity and contents of hydroxyproline (Hyp) of the collagen subfractions, i.e., neutral salt soluble (NSC), acid soluble (ASC) and insoluble collagens (ISC) in comparison with histologic observation of the proliferation of connective tissue in the canine heart. The contents of Hyp in NSC and ASC increased in the infarcted tissue 5 days after coronary ligation prior to its increase in ISC. The content of Hyp in NSC, which is a precursor of ASC and ISC, reached the maximal value at 7 days and that reached the maximal value in ASC 7 to 14 days after coronary ligation. ISC increased markedly after 14 days. PHase activity increased on the second day and attained to the highest at 5 to 7 days. Proliferation of fibroblasts paralleled with the increases in PHase activity and NSC, and an increase in collagen fiber did with the contents of ISC. From these results it is concluded that in the healing stage of myocardial infarction, PHase was activated earlier than the increase in immature collagen in the infarcted area which was observed 5 to 7 days after coronary ligation and was followed by the production of mature collagen after 14 days.

IT has been recognized by many histologic examinations¹⁻⁶ that increased deposition of collagen fibers and the resulting fibrosis are associated with the production of connective tissue in the healing process of myocardial infarction. Many previous studies have been carried out mainly by the morphologic examination; however, the number of papers viewed from the biochemical aspect are small. Hy-

droxyproline (Hyp) is well known as a specific constituent of collagen, and its content in collagen has been corroborated to be constant in mammals.⁷ Therefore biochemical determination of Hyp makes it possible to estimate collagen content in the heart muscle. The biosynthesis of collagen involves several unique enzymatic steps. One of these is the catalysis by prolylhydroxylase (PHase) which converts certain proline residues, already in peptide linkage of proto-collagen, to Hyp residues.⁸ It is also a well known fact that collagen in fibroblasts is required for the maturation process from the immature to the mature collagen fibers which play a main role in the growing connective tissue. In this study the proliferative process of connective tissue in the

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healing stage of experimental myocardial infarction was performed by the determinations of PHase activity and the contents of Hyp of three collagen fractions existing in growing connective tissue, i.e. the immature and mature collagens. And these results are compared to the morphologic observations of the infarcted tissue.

MATERIALS AND METHODS

Animal experiments: Myocardial infarction was produced in 48 adult mongrel dogs weighing 12 to 26 kg by the method described previously.⁹ Briefly, after anesthesia with intravenous injection of sodium pentobarbital (Nembutal®) at 30 mg per kg body weight, the dog was intubated, ventilated with room air with a Harvard respirator, and subjected to a left thoracotomy at the 5th intercostal space. The left anterior descending coronary artery was isolated from fatty tissue and ligated totally just below the first diagonal branch with a silk thread. Production of myocardial infarction was confirmed by continuous ST elevation followed by abnormal Q waves in electrocardiogram. At 24, 48, and 72 hours, 5, 7, 14, 21, and 28 days after coronary ligation, 4 to 5 dogs were subjected under anesthesia with Nembutal. The heart was removed in the beating state and the blood was washed out immediately with 0.9% NaCl and placed on ice.

Preparation of samples: Approximately 2g of the central portion of infarcted left ventricular anterior heart muscle was removed as a sample and the same amount of the non-ischemic myocardium of the posterior wall was treated as a control. All the procedures were carried out at 4°C unless otherwise mentioned. Both specimens were minced with scissors and homogenized with Polytron tissue processor in 9 volumes of 0.1 M NaCl and 0.005 M NaHCO₃ solution. The homogenate was centrifuged at 9,000 × G for 10 min. PHase activity was assayed in the supernatant. The precipitate was suspended in the same solution and was centrifuged again. This procedure was repeated five times until all protein was absent in the supernatant. The final precipitate was used for the extraction of collagens and the quantification of contractile proteins. One gram wet weight of the precipitate was placed in a flask and suspended in 30 ml of 0.45 M NaCl and 0.05 M tris-HCl (pH 7.4) for 48 hours with continuous stirring. After centrifugation at 8,000 × G for 10 min, the super-

natant was reserved and was designated as the fraction containing neutral salt soluble collagen (NSC). The precipitate was suspended again in 30 ml of 0.5 N acetic acid solution and was stirred for 48 hours. The supernatant of 8,000 × G centrifugation of this suspension was designated as the fraction containing acid soluble collagen (ASC), and the final precipitate was regarded as the residue fraction (insoluble collagen; ISC). Meanwhile, the contents of contractile proteins in the initiating precipitate was implicitly assumed to be non-collagen-proteins.

Determination of hydroxyproline: One to two ml of each fraction was placed in a test tube and hydrolyzed at 110°C for 18 hours in 6 N HCl by the addition of 12 N HCl. After hydrolysis, the Hyp content was determined by the method of Kivirikko and Prockop.¹⁰

Prollyhydroxylase activity: PHase activity of 9,000 × G supernatant of myocardial homogenate was assayed essentially according to the method of Hutton et al.^{10,11}

Each 1.0 ml assay mixture contained 200 μmoles tris-HCl buffer (pH 7.5), 0.2 μmoles FeSO₄·7H₂O, 2 μmoles sodium ascorbate, 4 μmoles α-ketoglutarate, 50 μg catalase, 100 μl peptidyl-(4-H³)-proline labelled substrate (107 × 10⁴ DPM/ml), 400 μl enzyme sample and 10 μmoles reduced glutathione. The substrate was prepared by incubating minced rat fetuses with 4-H³-proline in the presence of dipyrindyl, and by following extraction of labelled protocollagen according to the method of Hutton.¹² Radioactivity was determined in a Packard type 3255 TRI-CARB liquid scintillation spectrometer, and the substrate exhibited the radioactivity of 107,000 DPM/0.1 ml.

Non-collagen-proteins: Non-collagen-proteins were assayed according to the method of Sugita.¹³

Histologic examination: Infarcted heart tissue blocks were fixed in buffered formalin and stained with hematoxylin and eosin and with Mallory's stain, and observed under an oilimmersion lens.

Statistical analysis was performed by Student's t-test, and p values of less than 0.05 were considered significant.

RESULTS

1. Hydroxyproline contents of collagen fractions in the infarcted myocardium

Fig.1. Hydroxyproline contents in the neutral salt soluble and acid soluble collagen in the infarcted canine heart. Bars indicate mean $\pm$ S.D. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ against control value.

Fig.2. Hydroxyproline contents in the insoluble collagen and whole homogenate in the infarcted canine heart. Bars indicate mean $\pm$ S.D. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ against control value.

(1) Hyp contents in NSC and ASC fractions

Figure 1 shows Hyp contents in NSC and ASC fractions. In the non-ischemic myocardium, Hyp contents in NSC and ASC fractions were low, on

the average 0.59 and 0.64 $\mu\text{moles/g}$ wet weight, respectively. In the infarcted myocardium, no changes in their contents were observed until 72 hours after coronary ligation. However, on the

Fig. 3. Prolylhydroxylase activities in the infarcted canine heart. Bars indicate mean $\pm$ S.D. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ against control value.

Fig. 4. Contents of structural proteins in the infarcted canine heart. Protein concentration was determined by biuret method. Bars indicated mean $\pm$ S.D. *** $p < 0.001$ against control value.

5th day, the content of Hyp increased significantly in NSC ($p < 0.01$) and in ASC ($p < 0.05$). The content of Hyp in NSC, increased by 4 times ($2.40 \mu\text{moles/g}$ wet weight on the average) as much as control value ($0.59 \mu\text{moles/g}$ wet weight) at 7 days after ligation, and decreased thereafter, but it was still twice as much as

control level on the 28th day. The content of Hyp in ASC increased to $1.14\text{--}1.17 \mu\text{moles/g}$ wet weight ($p < 0.001$) after 7 to 14 days, and attained at the maximal content, twice as much as control ($0.69 \mu\text{moles/g}$ wet weight), and decreased thereafter. The rate of increase in Hyp content in ASC was less than that in NSC.

Fig.5. Photomicrographs of 3, 5 and 7 days after coronary ligation in the dog. A: 3 days after coronary ligation, B: 5 days after coronary ligation, C: 7 days after coronary ligation. The upper part; Hematoxylin and eosin $\times 250$ The lower part; Azan-Mallory $\times 250$

(2) Hyp contents in ISC and whole homogenate

Figure 2 shows Hyp contents in ISC, designated as the residue fraction, and in whole homogenate. In the non-ischemic myocardium, the Hyp contents in ISC and in whole homogenate were on the average 6.04 and 8.46 $\mu\text{moles/g}$ wet weight, respectively. The Hyp content in NSC decreased initially, 48 to 72 hours after ligation, to 58% of the control level. However, after 7 days it returned to the control level and then increased markedly to 3 times as much, (20 $\mu\text{moles/g}$ wet weight) as the control ratio 28 days after coronary ligation. In whole homogenate, the alteration was very similar chronologically to that of ISC, because the greater part of them was occupied by ISC. The content decreased to 60% of the control level 48 to 72 hours after coronary ligation, and thereafter increased markedly from 7 to 28 days to 3.6 times (29.6 $\mu\text{moles/g}$ wet weight), as the control level.

2. Prolylhydroxylase activity in the infarcted myocardium

Figure 3 shows PHase activity in the infarcted myocardium. PHase activity of $9,000 \times G$ supernatant, exhibited an average of 3,600 DPM/g wet weight in the non-ischemic heart

muscle and increased significantly ($p < 0.001$) in the infarcted myocardium on the second day prior to increases in NSC, ASC and ISC, and the peak was 29,000 to 32,000 DPM/g wet weight and occurred 5 to 7 days after ligation. The activity decreased on the 28th day, but it was still twice as much as the control ratio.

3. Contents of structural proteins in the infarcted tissue

Figure 4 shows the contents of non-collagen proteins in structural proteins. The majority of the proteins was occupied by contractile proteins. In the infarcted tissue, the protein content began to decrease 48 to 72 hours after coronary ligation, to about 30% of that of control at 7 days. The contents recovered to the control level gradually on the 21st to 28th day. The chronological changes in contractile proteins were quite similar to those in the report of Katagiri.¹⁴

4. Histologic findings

Figure 5 shows photomicrographs of infarcted cardiac tissues. Two to 7 days after coronary ligation, the cardiac muscle fibers exhibited the feature of the typical coagulation necrosis. They were stained intensely eosinophilic with swelling, and abundant fragmentation and segmentation were observed. The extension and loss of cross-

striation were found in places. Neutrophil polymorphonuclear leucocytes increased rapidly in number and mass between the degenerated muscle fibers. They were more numerous from the 3rd to 5th day after ligation. The observed histologic changes were similar to those described by many previous authors.¹⁻⁶ With respect to fibroblasts, they could first be seen in the infarcted tissue on the 2nd to 3rd day after ligation. Their nuclei were ovoid, pale and large. The cytoplasm was basophilic and abundant. Those young full fibroblasts, as seen under a light microscope, seemed to be actively synthesizing collagen. But collagen fibers were not observed around these young fibroblasts. Five days after ligation, small collagen fibers appeared in the extracellular space, but they were not stained blue with Mallory's stain. Those were thought to be immature collagen fibers. On the 7th day, collagen fibers increased moderately and these fibers were stained deeply blue and indicated their maturation.

DISCUSSION

Biochemical studies on collagen in the field of cardiac diseases have been carried out mainly in hypertrophic heart muscle,¹⁵⁻¹⁸ but those in myocardial infarction are few. Almost of such studies were of the histopathologic ones, in which collagen fibers in the granulation tissue were examined by Mallory's stain or silver impregnation.²⁻⁶

It was established that in myocardial infarction the biochemical determination of collagen content was paralleled with pathologic changes.^{19,20} O'Donnell et al. examined the normal and ischemic canine hearts and observed that increased ultrasonic attenuation correlated well with increased concentration of collagen which was determined biochemically in relation to ischemic injury 2, 4, and 6 weeks after coronary occlusion.²¹⁻²³ Akagami and Shibata described that Hyp contents in the infarcted heart muscle decreased from 2 to 7 days after coronary ligation in the dog, and increased rapidly at 10 days.²⁴ However, few studies have been performed concerning the synthetic process of collagen in the degrading myocardium under ischemia, i.e. the maturation process from immature collagen fibers to functioning fibers. Meanwhile, the histologic studies on the healing process of myocardial infarction have been performed by many investigators.^{3,5,6} In short,

an infarcted tissue undergoes a process of healing, consisting of removal of the necrotic muscle fibers, and replacement by connective tissue which in time forms a firm, contracted fibrous scar. Eventually beginning on the 4th to 5th day and starting peripherally, new capillaries grow into the infarcted tissue accompanied by cell elements of connective tissue responsible for most collagen formation. With the ingrowth of new capillaries and fibroblasts, macrophages invade and phagocytize the necrotic tissue 5 to 7 days after the onset of infarction. Newly formed collagen fibers, produced by fibroblasts, are usually produced first after 7 days and are thereafter conspicuous for 2 weeks. The healing process of infarct in the dog heart is similar in most respects to that of human heart, except that the process is faster.

From a biochemical viewpoint, it is generally accepted at present that the biosynthesis of collagen is carried out by a series of sequential steps, i.e. the assembly on ribosomal complexes of polypeptide precursor of collagen that have been called "procollagen", the hydroxylation of appropriate proline and lysine residues in procollagen to Hyp and hydroxylysine, and the substitution of some of the glycosidic linkage before the molecule is extruded. Procollagen is converted to the molecular structure referred to as "tropocollagen" by some proteases which are not completely identified yet. Collagen fiber is formed by cross-links between adjacent tropocollagen molecules. Collagen which is synthesized in such a fashion as described above, has been known to exist in three forms, i.e. neutral salt soluble collagen, acid soluble and residue collagens which is synonymous with insoluble collagen. The NSC fraction represents the youngest form of the collagen precursor into ASC and ISC.^{25,26} As mentioned above, NSC, which has been known as reticulin, is the most immature type of collagen fiber.

Although collagen growth in the healing wound has been investigated intensively in histological studies, this article may be the first report on biochemical determinations of Hyp contents in soluble and immature collagens together with PHase activity in acute myocardial infarction before collagen fibers are visible histologically.

On the characteristics of cardiac muscle collagen, it is totally intermolecularly cross-linked and the yield of ASC and NSC from rat cardiac collagen were only 0.1-1.0% of that

Fig.6. Time table of histologic and collagen changes in the infarcted canine heart.

obtained from rat skin collagen.²⁷ Meanwhile, the proportion of NSC Hyp in the myocardium dropped from an initial value of 30% of the total Hyp in newborn rats to 10% at the age of three weeks.²⁸ Then the proportion of soluble collagen remained almost unchanged.²⁸ In this study, the Hyp content of soluble collagens, NSC plus ASC was 16% of the total collagen content in the normal myocardium of the mongrel dog.

The time course of events in the healing of wounds has been reviewed. During the first productive state of healing which lasts 4 to 5 days, there is a rapid increase in the number of fibroblasts as a result of cell migration and division, but no increase in collagen, as measured by saline extractable Hyp.²⁹ According to Dunphy et al., an increase in extractable Hyp first appears on the 4th to 5th day, which is a day or two later than the increase in PHase.³⁰ Stein et al. reported that in granulating wounds of rats and humans the PHase activity elevated on the second day and exhibited the maximal value at 4 to 5 days, and NSC increased on the 5th day.³¹ In this study the process of the production of collagen in myocardial infarction is similar to that in wounds, except for decrease in collagen contents in the early necrotized stage of the infarction.

Eventually, NSC and ASC increased significantly 5 days after coronary ligation, then fibroblasts increased more and collagen fibers, which were like reticulin histologically, began to be stained with anilin around fibroblasts. The maximal value of NSC was exhibited at 7 days, and ASC was at 7 to 14 days. As the mature

collagen fibers were stained blue in Mallory's stain, the ISC was increased markedly around the 14th day. The rate of increase in ASC was less than that in NSC. It is conceivable that the turnover rate of ASC to ISC was so rapid and constant that an increase in ASC could not be detected. The decrease in Hyp content in ISC 48 to 72 hours after coronary ligation is attributed to the necrosis of the collagen fiber in the infarcted myocardium, therefore soluble collagen fractions (NSC and ASC) increased at this time. Meanwhile PHase activity of soluble fraction of the infarcted tissue increased significantly on the second day, at that time fibroblasts appeared in the infarcted area. Its activity increased markedly with the proliferation of fibroblasts, and it exhibited the peak activity at 5 to 7 days prior to the peak in NSC. The most practical method of stimulating PHase activity is by ascorbic acid which is one of the cofactor of PHase. Collagen metabolism will become more rapid by an administration of large amount of ascorbic acid in the recovery stage of myocardial infarction, and necrotic tissue might be replaced by collagen fibers more rapidly. The decrease in non-collagen-proteins in the sedimentative fraction, most of which is constituted of contractile proteins, corresponded to the chronologic alterations in myocardial necrosis.⁹ These changes in biochemical and morphologic results are summarized in Figure 6.

From these results, it is concluded that in the recovery stage of myocardial infarction PHase is activated earlier, and then immature collagens (NSC and ASC) increase 5 to 7 days after the

onset of myocardial infarction followed by the production of mature collagen (ISC) after 7 to 14 days.

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