A STUDY ON MYOCARDIAL FIBROSIS IN MYOCARDIAL INFARCTION AND IN IDIOPATHIC CARDIOMYOPATHY: A MEASUREMENT OF HYDROXYPROLINE LEVEL IN PLASMA AND IN MYOCARDIUM

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Development of fibrosis as recovering process after a tissue injury could be one of the important factors to impede functions of organs involved. Especially in the heart, the fibrosis decreases diastolic compliance and have effects on synergy in systole. In idiopathic cardiomyopathy, it is well known that there occurs myocardial fibrosis with unknown causes followed by cardiac dysfunction. The author attempted to study dynamics of tissue fibrosis in the heart and blood vessels in the experimentally produced myocardial fibrosis and the patients with cardiovascular disease.

SUBJECTS AND METHODS

Protein bound hydroxyproline (H.O.P.) levels in the plasma were measured in 11 cases of healthy adult, 31 cases of idiopathic cardiomyopathy [hypertrophic obstructive cardiomyopathy (HOCM): 4 cases; congestive cardiomyopathy (CCM): 27 cases], 2 cases of endocardial fibroelastosis, 14 cases of myocardial infarction, 6 cases of Takayasu’s arteritis, 6 cases of Marfan’s syndrome, and 3 cases of dissecting aneurysm of aorta.

For experimental myocardial infarction, 14 adult dogs weighing 12–15 kg of body weight were used. For experimental myocardial necrosis, 48 rats of Wistar strain weighing 150–200 g of body weight were used.

The experimental myocardial infarction in dogs: Animals were anesthetized with pentobarbital and the chest was opened and the anterior descending branches of left coronary artery were ligated between the first and the second branches to produce myocardial infarction. Then the animals were sacrificed on the fifth, tenth, twentieth, fiftieth, seventieth, and ninetieth day respectively after the ligation with electrocardiogram recorded before sacrificed. The hearts were excised and infarcted areas were used as specimen for microscopic examination with Hematoxylin-Eosin stain and Azan or Masson stain, and were also used to measure total H.O.P. in the myocardium.

Experimental myocardial necrosis in rats: For necrotizing rat-myocardium, 5 mg/kg of isoproterenol was injected into the peritoneal cavity daily for 10 successive days and the rats were sacrificed 1 week, 2 weeks, 1 month, 3 months, 6 months, 9 months, and 12 months after the injection respectively. H.O.P. in the plasma and the myocardium were measured and microscopic examinations of the myocardium were performed.

Measurement of H.O.P. in the plasma: A method by Procop and Udenfriend was used. The plasma was separated from the blood and 4 ml of saturated Ba(OH)₂ solution was added to it and was placed in an autoclave with a temperature of 124°C for 16 hours to hydrolyze. After the hy-
hydrolysis, the solution was oxidized by chloramin T to convert hydroxyproline into pyrrole-2-carboxylic acid. Ion exchange resin of AGI-X8 and 200-400 mesh chloride form was used to purify the solution and the solution was heated at 100°C for 30 minutes to change pyrrole-2-carboxylic acid into pyrrole. Then, the solution was mixed with toluene to transpose all pyrrole into the toluene. A part of the toluene solution was stained by adding Ehrlich's reagent and the pyrrole level in the toluene solution was measured by a spectrophotometer (HITACHI PERKIN-ELMER 139UV-VIS Spectrophotometer) using a wave length of 560 mu.

Measurement of H.O.P. in the myocardium: The myocardium was dried at a temperature of 100°C for 3 days. The dry sample was powdered and weighed and mixed with 6 N hydrochloride solution. The solution was put into autoclave with a temperature of 124°C for 10 hours. Then the solution was filtered with a Whatman No. 1 filter paper. With the same method as in plasma H.O.P. measurements, H.O.P. in the filtrate was converted into pyrrole and the pyrrole was measured by a spectrophotometer.

H.O.P. concentration was expressed in mcg/mg of dry heart weight. The value achieved by the method represents protein bound hydroxyproline level in the plasma and total hydroxyproline level in the myocardium. The chemical recovery rate by the method above mentioned was 70 per cent.

To express numerically a grade of myocardial fibrosis, the specimen stained by Azan or Masson staining was examined microscopically by a following method.

A microscopic image was projected and enlarged 360 times on a screen with 10 cm by 10 cm, and 300 points, where scale lines were crossing, were checked for fibrosis focus. A frequency of the fibrosis foci was expressed in per cent and the value was used as an index to show a grade of myocardial fibrosis.

To demonstrate a density of fibroblast in the myocardium, point system was used. When a number of fibroblast seen in an area of 1 mm² of microscopic specimen was between 1 and 60, 1 point was given, and 2 points for a number of fibroblast between 61 and 120, 3 points between 121 and 180, and 4 points between 181 and 240. Next, the points from four areas of 1 mm² of the microscopic specimen were added up and the total point was used as a fibroblast index.

RESULTS

Experimental myocardial infarction in dogs.

Five days after the ligation of anterior descending branch of left coronary artery, the electrocardiogram showed an elevation of the ST segment in Leads V₁ and V₄, but on the 20th and 90th day, there was no elevation of the ST segment. On the 90th day, the electrocardiogram revealed inverted T waves in V₄ and V₅. A microscopic examination of the infarcted area with Hematoxylin-Eosin staining was performed. Five days after the infarction, there was mainly round cell infiltrations and fibroblast was rare, and on the 20th day a small number of round cell infiltration and a large number of fibroblast were recognized. On the 90th day, both round cell infiltration and fibroblast almost disappeared. Figure 1 illustrates change of myocardial H.O.P. level, fibroblast index, and grade of fibrosis. Within five days after the infarction, there was no increase in fibroblast, but on the 10th day, fibroblast index was at the highest point of 5 and gradually decreased and returned within normal limit after 70 days. Fibrosis was hardly observed on early days after the infarction, and was 6 per cent on the 5th day and 7 per cent on the 10th day. But on the 20th day the grade of fibrosis increased markedly to 18 per cent and
Myocardial Fibrosis in Myocardial Infarction

Fig. 2. Photomicrograph of the Azan stained myocardium of a rat which sacrificed 12th month after 10 days consecutive injections of isoproterenol intraperitoneally (× 30).

Fig. 3. The changes of the myocardial H.O.P. level, the fibroblast index, and the plasma H.O.P. level in experimental rat. N: normal rat.

reached to 20 per cent on the 50th day, and remained unchanged thereafter. The level of H.O.P. in myocardium of healthy dog was 5.45 ± 0.31 mcg/mg (mean ± SD), and was 5.6 mcg/mg and 5.8 mcg/mg on the 5th and 10th day after the infarction respectively. On the 20th day it increased rapidly to 26 mcg/mg and showed no changes thereafter. That is, after increase in number of fibroblast in the myocardium, the level of H.O.P. in the myocardium and the grade of fibrosis remained unchanged.

Experimental myocardial necrosis and fibrosis.

Figure 2 demonstrates the Azan stained myocardium of a rat which was sacrificed on 12th month after 10 days successive injection of isoproterenol intraperitoneally. There were foci of marked myocardial fibrosis, especially in the inner one third layer of the myocardium. Figure 3 showed the changes of the myocardial H.O.P. level, the fibroblast index, and the plasma H.O.P. level. The myocardial H.O.P. level was 4.7 ± 0.7 mcg/mg in healthy rats and was 2.7 ± 0.3 mcg/mg and 3.2 ± 0.5 mcg/mg on the first and 2nd week respectively after the myocardial necrosis, which showed temporal decrease. The level turned into increase thereafter until the 1st month and was 5.8 ± 1.4 mcg/mg. This tendency to increase the H.O.P. level continued until the

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6th month and the level was $9.0 \pm 3.0$ mcg/mg. The level on the 9th month and 11th month was $9.3 \pm 2.2$ mcg/mg and $11.4 \pm 3.4$ mcg/mg respectively which showed slight increase. The index of fibroblast started to increase one week after the isoproterenol injection and continued to increase until 3 months and remained unchanged after that. The plasma H.O.P. level of healthy rats was $7.9 \pm 1.3$ mcg/ml. The level increased to $9.8 \pm 2.5$ mcg/ml one week after the injection and reached to $11.1 \pm 2.1$ mcg/ml one month later. Thereafter, there was tendency to decrease but still remained at high level of $9.5 \pm 2.3$ mcg/ml 12 months later. Figure 4 illustrates a relationship between the H.O.P. levels and the grade of fibrosis in the rat myocardium after the isoproterenol injection. There was positive relationship and the correlation coefficient $r$ was $0.790$ ($P < 0.05$).

Plasma H.O.P. levels in cardiovascular diseases.

As demonstrated in Figure 5, the plasma H.O.P. level of eleven of healthy human ranged from $3.5$ mcg/ml to $6.2$ mcg/ml and the mean value was $4.9$ mcg/ml. Out of 27 patients with congestive cardiomyopathy, only four patients showed normal H.O.P. level and seven showed plasma H.O.P. level of $7.0$ mcg/ml or more. With hypertrophic obstructive cardiomyopathy, all showed high

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**Fig. 4.** Relationship between the H.O.P. level and the grade of fibrosis in the rat myocardium after isoproterenol injection.

**Fig. 5.** Plasma H.O.P. levels in cardiovascular diseases.

Abbreviations: ICM=idiopathic cardiomyopathy, COCM=congestive cardiomyopathy, HOCM=hypertrophic obstructive cardiomyopathy, EFE=endocardial fibroelastosis, IHD=ischemic heart disease.
value and three out of four patients showed 7.0 mcg/ml or more. Two patients with endocardial fibroelastosis showed slightly high level of 6.4 mcg/ml and 6.6 mcg/ml respectively. Regarding myocardial infarction, plasma H.O.P. level two weeks after attack was taken as a level for fresh myocardial infarction. Myocardial infarction one year or longer after attack was taken as old myocardial infarction. With fresh myocardial infarction, five out of six patients demonstrated abnormal high H.O.P. peak value of 10 mcg/ml or more. On the other hand, with old myocardial infarction, only one patient showed slightly high value of 6.4 mcg/ml. With ischemic heart disease except myocardial infarction, all five patients showed normal value. With Marfan's syndrome, all cases except one showed high level and the highest value was 14.4 mcg/ml. Five out of 6 patients with Takayasu's arterities showed abnormal high level of 8.4 mcg/ml or more. With dissecting aneurysm of the aorta, all three patients showed marked high level of 10 mcg/ml or more. In a patient with dissecting aneurysm of the aorta whose plasma H.O.P. level was measured repeatedly, the levels were 17.4 mcg/ml and 16.6 mcg/ml one week and one month.

<table>
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<th>Weeks after attack</th>
<th>0~2</th>
<th>~4</th>
<th>~6</th>
<th>~10</th>
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<td>11.02</td>
<td>8.18</td>
<td>5.83</td>
<td>5.22</td>
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<td>1.74</td>
<td>1.19</td>
<td>0.58</td>
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$n = 14$

Fig. 6. A case of fresh myocardial infarction.
after attack respectively and stayed at high level of 14.5 mcg/ml three months later with very gradual decrease.

The change of plasma H.O.P. levels in patients after myocardial infarction.

The change of plasma H.O.P. levels in patients after myocardial infarction was illustrated in Table I. Plasma H.O.P. level begun to increase one week after attack and reached to 11.0 ± 1.7 mcg/ml of average maximum value between two and four weeks, and decreased to 8.2 ± 1.2 mcg/ml between four and six weeks and returned to normal level of 5.8 ± 0.6 mcg/ml. In case of old myocardial infarction one year or longer after attack, plasma H.O.P. level was within normal limits, namely 5.2 ± 0.7 mcg/ml. Figure 6 demonstrated plasma H.O.P. level with some clinical findings in one case of fresh myocardial infarction. ECG showed marked ST segment elevation on the first day. On the third week,

Fig. 7. The changes of fibroblast index in myocardium after infarction in patients and in dogs. ○ ○ ○: mean value of six patients who survived longer than one year after infarction.

Fig. 8. Relationships between plasma H.O.P. level, fibroblast index and years after onset of symptom in idiopathic cardiomyopathy.
coronary T wave was seen but there was no marked change in other findings of ECG since then. Regarding serum enzymes such as CPK, LDH and GOT, all returned to normal value completely on the third week. On the contrary, the plasma H.O.P. level was at its maximum on the third week when the serum enzyme levels returned to normal value, and the level turned to decrease thereafter to 10.0 mcg/ml and 9.2 mcg/ml on the fourth and fifth week respectively and to normal value on the seventh week.

The change of fibroblast index in myocardium after infarction.

Figure 7 shows a process of intramyocardial fibroblast appearance in patients and in dogs after infarction. It has been demonstrated at autopsy both in patients and in dogs with myocardial infarction that fibroblast developed very few soon after infarction and increased markedly 10 and 20 days after infarction and gradually turned to decrease thereafter. In one patient who died and was autopsied on 60th day after infarction, there was still an increased number of fibroblast. In other six patients who survived longer than one year after infarction, there was no increase of fibroblast in all cases.

Plasma H.O.P. level in idiopathic cardiomyopathy.

Figure 8 demonstrated relationships between plasma H.O.P. level, intramyocardial fibroblast index and years after onset of symptom. In idiopathic cardiomyopathy, there was no decrease of plasma H.O.P. level in course of time and the level was high in either cases with short or long history. In six out of seven cases who progressed longer than ten years after onset of the symptom, plasma H.O.P. level ranged from 6.3 mcg/ml to 8.4 mcg/ml and the average value of all seven cases was as high as 6.8 mcg/ml. About fibroblast index, many showed high value unrelated to the length of history as same as plasma H.O.P. level, and eight out of ten cases within one year after onset of symptom showed high value. All cases who died with a history of two years or longer after onset of symptom also showed high value. In five cases, plasma H.O.P. levels were measured repeatedly in the course of disease and those changes were illustrated in Figure 9. In patient F.T., plasma H.O.P. level was 10.2 mcg/ml at the first time and remained high after nine months with slight decrease. In patient T.Y., the value were 9.0 mcg/ml at the first time, and 9.1 mcg/ml and 6.5 mcg/ml six and twelve months later respectively, which showed a gradual decrease same as in F.T. case. During those periods, clinical signs of both patient F.T. and patient T.Y. were relatively stable and showed arrhythmias only. In patient K.A., plasma H.O.P. levels fluctuated and were 8.2, 15.7, 11.2 5.6, 4.9, and 7.0 mcg/ml. Maximum level was achieved at the second time and there was frequent ventricular extrasystoles and occasional Adams-Stokes attacks at this time. In patient M.Y., plasma H.O.P. level was 7.4 mcg/ml initially, 7.7 mcg/ml second time, and 7.8 mcg/ml third time, which showed a tendency to increase but there were no remarkable change of clinical signs. In patient K.Y., initial value was 6.8 mcg/ml, second time 8.3 mcg/ml, and third time 9.6 mcg/ml which showed a tendency to increase. During this period, the patient had a change for the worse with frequent arrhythmia and severe cardiac decompensation, and cardio-thoracic ratio increased remarkably.

DISCUSSION

Generally speaking, after a tissue injury, granulation tissue proliferates and fibrosis follows. When fiber protein is synthesized, fibroblast takes proline in from blood and synthesize hydroxyproline to excrete as tropocollagen which polymerize to form fiber in combination with cross-linkage of collagen fiber. Just after fiber formation, fresh collagen, which is so-called soluble collagen, gradually changes into insoluble hard collagen. In this study, by measuring H.O.P. level in plasma and in myocardium, the author tried to study dynamics of
collagen metabolism after tissue injury in cardiovascular disease, especially of myocardial fibrosis in myocardial infarction and in idiopathic cardiomyopathy.

(I) Inquiry for hydroxyproline measurement.

To measure H.O.P. level in plasma and in tissue, many methods have been reported,11,12,13,14,15,16,17,18

In this study, H.O.P. level was measured by the method reported by Prockop and Udenfriend19 in which H.O.P. is determined by measuring pyrrole into which H.O.P. is converted. It is able to measure plasma H.O.P. even as low as less than 2 mcg/ml by this method which can provide reliable result. For drying myocardium to measure H.O.P. level in it, myocardium was put in a dryer with 100°C for three days. With one day drying, myocardium specimen weight reduced to twenty per cent of initial wet weight and there was no change in weight thereafter. In a report by Buccino,20 average H.O.P. level in epicardial side of cat myocardium was 7.9 mcg/ml. In this study, average for myocardial H.O.P. in epicardial side of the left ventricle in dogs was 7.8 mcg/ml which was as nearly as the former value even with different experimental animal.


In experimental myocardial necrosis with rat, fibroblast began to increase in number one week after isoproterenol injection intraperitoneally, and continued to increase for three months. Plasma H.O.P. level started to increase one week after the injection when fibroblast appeared, and reached to its maximum level one month later and remained at this level thereafter, which showed that an increase of plasma H.O.P. was accompanied with an increase of fibroblast. That is, plasma H.O.P. level is reflecting dynamics of fibroblast in tissue. As mentioned before, plasma H.O.P. level in some cardiovascular diseases showed high value. This indicates that granulation and fibrosis in myocardium and/or in arterial wall are ongoing in such diseases as idiopathic cardiomyopathy, endocardial fibroelastosis, fresh myocardial infarction, Marfan's syndrome, Takayasu's arteritis, and dissecting aneurysm of the aorta.

(III) Plasma H.O.P. in myocardial infarction.

Release of myocardial enzymes into the blood stream after myocardial infarction soon reached to its maximum values and normalized by fourth week at latest.

On the contrary, collagen formation as a recovery covering mechanism in injured myocardial tissue was delayed, and within one week after attack fibroblast in myocardium was small in number and plasma H.O.P. level was also low. After that, both fibroblast and H.O.P. level started to increase and the latter reached to maximum level between two and four weeks after attacks and gradually decreased after that. Those results indicate that within one week after myocardial infarction, granulation and fibrosis are poor and this period is considered to be dangerous for ventricular rupture21,22,23 and collagen formation activity in the myocardium is high until one month after attack and decrease thereafter, and goes into a phase of tissue reinforcement by collagen polymerization.

(IV) Plasma H.O.P. level in idiopathic cardiomyopathy.

With idiopathic cardiomyopathy, some patients showed high plasma H.O.P. level unrelated to a length of history and out of seven cases who progressed longer than ten year after the onset of symptoms, six showed high levels. Regarding fibroblast index, three cases who progressed seven, eight, and nine years after the first symptom showed high index number of five, six, and nine respectively. Those results suggest that in idiopathic cardiomyopathy myocardial fibrosis is always ongoing.

CONCLUSION

Myocardial necrosis was experimentally produced by injecting isoproterenol intraperitoneally in rat and myocardial infarction was made by ligating anterior descending branch of left coronary artery in dog. Studies were performed on changes of fibroblast and a grade of fibrosis in myocardium, and H.O.P. levels in plasma and myocardium were measured. Plasma H.O.P. levels in patients with cardiovascular disease were measured and results were as follows;

1) Fluctuations in plasma H.O.P. level are reflecting appearance and disappearance of tissue fibroblast, and therefore by measuring plasma H.O.P. level, it is possible to presume the dynamics of tissue fibrosis.

2) Plasma H.O.P. level in healthy human ranged from 4 to 6 mcg/ml. Average values for following diseases were high as follows; dissecting aneurysm of aorta: 13.9 mcg/ml, fresh myocardial infarction: 13.4 mcg/ml, Takayasu's arteries: 10.0 mcg/ml, Marfan's syndrome: 8.2 mcg/ml, hypertrophic obstructive cardiomyopathy: 7.9 mcg/ml, and congestive cardiomyo-

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pathy: 7.3 mcg/ml.

3) In myocardial infarction, plasma H.O.P. level was at its highest point between two and four weeks after attack and returned to normal between seven and ten weeks. Those results indicate that collagen formation in an infarcted area is most active between two and four weeks after attack. Collagen formation activity decreases to normal seven to ten weeks after attack and tissue reinforcement in the area develops by collagen polymerization.

4) In idiopathic cardiomyopathy there was fibroblast in myocardium unrelated to the length of history and many patients showed high plasma H.O.P. level. From those findings myocardial fibrosis is considered to be progressive in this disease.

REFERENCES