HISTOLOGICAL AND HISTOMETRICAL STUDY OF MYOCARDIAL FIBROSIS IN SPONTANEOUSLY HYPERTENSIVE RATS OF THE STROKE-PRONE STRAIN

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In order to obtain fundamental information about the developmental mechanisms of myocardial fibrosis in chronic hypertension, the hearts of male spontaneously hypertensive rats of the stroke-prone strain (SHRSP) and Wistar rats of the Kyoto strain (WKY) were histologically and histometrically examined. Fibrosis was a prominent histological feature of the hearts in SHRSP. It consisted of focal, interstitial, and perivascular fibrosis.

For histometrical analysis the percentage areas of interstitial and perivascular fibrosis were calculated by using a color image processor. The percentage area of myocardial fibrosis increased with advancing age in both SHRSP and WKY. However, it was significantly higher in SHRSP than in WKY at 18 and 30 weeks of age. In SHRSP perivascular fibrosis of small arteries had already appeared at 8 weeks of age, while perivascular fibrosis of arterioles and interstitial fibrosis developed later. It is supposed that perivascular as well as interstitial fibrosis is induced by the exudation of some growth factors due to an increased vascular permeability. On the other hand, the focal fibrosis observed in old SHRSP is suspected to occur as a result of injury in myocardium due to stenosis or occlusion of vessels.

ALTHOUGH fibrosis is one of characteristic features of hypertensive heart disease¹⁻³ there has been no systematic pathological research on it, and the developmental mechanisms of fibrosis have not yet been clarified. Because human hearts in hypertension are accompanied, more or less, by lesions due to atherosclerosis in the coronary arteries, it is very difficult to analyze which lesions are really caused by hypertension. Spontaneously hypertensive rats of the stroke-prone strain (SHRSP)⁴ de-

rived from spontaneously hypertensive rats (SHR)⁵ show very high blood pressure and various organic changes in the heart, and can be regarded as a good animal model for studying pure hypertensive lesions². The purpose of the present study is to obtain fundamental information about the developmental mechanisms of myocardial fibrosis by investigating the hearts of SHRSP and Wistar rats of the Kyoto strain (WKY) as control animals sequentially according to ages. Myocardial fibrosis was examined histologically and histometrically using a color image processor.

Key words:

Spontaneously hypertensive rat
Hypertension
Heart
Fibrosis
Color image processor

MATERIALS AND METHODS

Groups of 8-, 18-, 30-week-old SHRSP

(Received August 17, 1989; accepted January 6, 1990)

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and age-matched WKY were used for the experiments. Each group consisted of 7 male rats. Some SHRSP died of cerebral bleeding due to severe hypertension, and such animals with overt complications were excluded from the present study.

The rats were weight, and the blood pressure was measured by the tail cuff, autpick-up method. The hearts were fixed at the end-diastolic state according to the Kuriyayashi procedure⁶ Under anesthesia by intraperitoneal injection of pentobarbital, the right nommon carotid artery was cannulated. After thoracotomy, 0.3 ml of a 7% solution of ethylenediaminetetraacetic acid 2 Na-salt, a potent chelating agent of calcium ion, was injected into the right atrium. The descending aorta was clamped with a small forceps, and the right atrium was incised for drainage. Immediately, the coronary artery was perfused retrogradely with 4% neutralized buffered formaldehyde through the cannulated carotid artery until the ventricular wall became pale. The perfusing pressure was

maintained at 80 mmHg. The heart was removed, blotted dry, and weighed. The left ventricle of each heart was cut evenly into 6 transverse slices, and immersed for several days in the same fixative. The tissue blocks were processed routinely for paraffin embedding. The blocks were sectioned at a thickness of 3 μ m. The sections were stained with Masson's trichrome and hematoxylin and eosin.

Blue-stained portions showing fibrosis in the Masson's trichrome preparations of the hearts were measured using a color image processor (Luzex 2D, Nikon). The processor consisted of a microsystem, RGB color television, camera, main processor, control computer, display, and data printer. The object glass in the microsystem was fixed at the magnification of $\times 10$.

Measurement was done on the transverse sections. Interstitial fibrosis was measured in the middle portion of the left ventricular free wall (LVF) and that of the interventricular septum (IVS) in the sections of the second slice from the apex (apex), as well as in the sections of the fifth slice from the apex (base). The ratio of the blue portions showing fibrosis in a rectangular domain, where there were no vessels except capillaries, to the domain itself was calculated, and was regarded as the percentage area of interstitial fibrosis (Fig. 1).

In the measurement of perivascular fibrosis, oval-shaped intramyocardial coronary arteries were selected. Perivascular fibrosis

Fig. 1. Schema of rectangular domain. 1=interstitial fibrosis

Fig. 2. A. Schema of vessel (transverse section)
 B. Schema of doughnut-like domain
 1=lumen; 2=perivascular fibrosis; 3=outer border of the media; 4=two-fold enlarged oval

TABLE I AGE, BLOOD PRESSURE, BODY WEIGHT, HEART WEIGHT, AND HW/BW

		BP (mmHg)		BW (g)		HW (g)		HW/BW ($\times 10^{-3}$)	
8W	WKY	111	(8)	202	(22)	0.92	(0.14)	4.55	(0.34)
	SHR-SP	163*	(5)	199	(13)	0.94	(0.12)	4.71	(0.39)
18W	WKY	130#	(11)	347	(11)	1.40	(0.11)	4.03#	(0.25)
	SHR-SP	226*#	(16)	259	(34)	1.33	(0.18)	5.17*	(0.55)
30W	WKY	124	(16)	395	(22)	1.49	(0.08)	3.79#	(0.22)
	SHR-SP	239*+	(17)	325	(21)	1.57	(0.08)	4.83*	(0.47)

Results are expressed as mean (SD).

*: Significant difference between SHRSP and WKY at the same age. $p < 0.01$

#: Significant difference from the rats of the same strain at 8 weeks. $p < 0.01$

+: Significant difference from the rats of the same strain at 18 weeks. $p < 0.01$

Fig.3. Perivascular fibrosis has already increased in SHRSP at 8 weeks. (Masson's trichrome stain, $\times 200$)

Fig.5. More prominent perivascular fibrosis was observed in SHRSP at 30 weeks. (Masson's trichrome stain, $\times 200$)

Fig.4. Prominent perivascular fibrosis was observed in SHRSP at 18 weeks. (Masson's trichrome stain, $\times 200$)

Fig.6. Perivascular fibrosis was not prominent in WKY at 30 weeks. (Masson's trichrome stain, $\times 200$)

of small arteries, 100 to 200 μm in external-diameter, and arterioles, 50 to 100 μm , was measured in the middle portion of the LVF in the apex sections, as well as the base sec-

tions. The term "arteriole" was used for the small arteries, ranging approximately from 50 to 100 μm in external diameter, having more than one smooth muscle layer and a

Fig.7. Interstitial fibrosis was not prominent in SHRSP at 8 weeks. (Masson's trichrome stain, $\times 200$)

Fig.9. More prominent interstitial fibrosis in SHRSP was observed at 30 weeks. (Masson's trichrome stain, $\times 200$)

Fig.8. Prominent interstitial fibrosis was observed in SHRSP at 18 weeks. (Masson's trichrome stain, $\times 200$)

Fig.10. Interstitial fibrosis was not prominent in WKY at 30 weeks. (Masson's trichrome stain, $\times 200$)

well-developed elastic interna. The ratio of the blue portions showing fibrosis in a doughnut-like domain, which was enclosed by a oval of the outer border of the media and the 2-fold enlarged oval, to the domain itself was calculated, and was regarded as the percentage area of perivascular fibrosis (Fig. 2). Because it is difficult to distinguish adventitia from perivascular pathological fibrosis, in this study the fibrosis of adventitia was included as perivascular fibrosis. Vessels with obvious branching or double vessels were excluded from the measurement of fibrosis.

The unpaired t-test was used to compare the percentage areas of fibrosis in SHRSP and WKY at the same age, and also those in either SHRSP or WKY at different ages, after the ratios of variances were tested. It was also used to compare either the blood

pressures or the ratios of heart weight to body weight (HW/BW) in SHRSP and WKY at the same age, and also those in either SHRSP or WKY at different ages. The paired t-test was used to compare the percentage areas of interstitial fibrosis in the LVF and IVS in each group, and also those in the apex and base. It was also used to compare the percentage areas of perivascular fibrosis of the small arteries in the apex and base in each group, and also those of the arterioles in them. Probability values of $p < 0.05$ were considered statistically significant.

RESULTS

The blood pressures of SHRSP were significantly higher from 8 to 30 weeks of age than those of WKY. The ratio of heart

weight to body weight (HW/BW) remained unchanged with aging in SHRSP, while the ratio in WKY decreased with advancing age. There were significant differences in the ratio between SHRSP and WKY at 18 and 30 weeks of age (Table I).

Coronary arterial walls showed thickening due to cellular hyperplasia and hyaline degeneration in the older SHRSP. Also, perivascular fibrosis accompanying adventitial cell proliferation was observed in SHRSP (Fig. 3–6). Some vascular smooth muscle cells contained very large nuclei.

Myocardial fibrosis in SHRSP increased with advancing age and consisted of focal fibrosis, interstitial fibrosis, and perivascular

Fig. 11. Focal fibrosis was observed in SHRSP at 30 weeks. Coronary arterial walls show thickening due to cellular hyperplasia. (Masson's trichrome stain, $\times 100$)

fibrosis. Interstitial fibrosis increased with advancing age (Fig. 7–10), and the focal fibrosis appeared from 18 weeks of age (Fig. 11). Perivascular fibrosis tended to be partly connected with interstitial fibrosis. In SHRSP, the degree of interstitial fibrosis and perivascular fibrosis seemed to increase from the outer to the inner third of the LVF. The hypertrophy of cardiocytes developed with aging in SHRSP, while this tendency was not observed in WKY. The valves, epicardium, and endocardium in SHRSP and WKY showed no remarkable changes.

The results of measurement of fibrosis by the color image processor were as follows. There was no significant difference between the LVF and IVS in the percentage area of interstitial fibrosis in each group (Fig. 12). Also, there was no significant difference between the apex and base in the percentage area of interstitial fibrosis (Table II). At 8 weeks of age, there was no significant difference between SHRSP and WKY in the percentage area of interstitial fibrosis in either the LVF or IVF. However, at 18 weeks, a significant difference appeared. At 30 weeks the difference became larger (Fig. 12).

On the other hand, at 8 weeks of age, the percentage area of perivascular fibrosis of the small arteries in SHRSP was larger than that in WKY, while there was no significant difference in the percentage area of perivascular fibrosis of the arterioles. At 18

Fig. 12. The percentage areas of interstitial fibrosis in LVF and IVS at different ages. There were no significant differences between the LVF and IVS in each group.
*: Significant difference between SHRSP and WKY at the same age. $p < 0.01$
*: Significant difference between SHRSP and WKY at the same age. $p < 0.05$

TABLE II PERCENTAGE AREAS OF INTERSTITIAL FIBROSIS IN THE APEX AND BASE

		Apex (%)		Base (%)	
8W	WKY	1.5	(0.7)	1.5	(0.2)
	SHR-SP	1.3	(0.9)	1.4	(0.2)
18W	WKY	1.9	(0.6)	2.2 [#]	(0.4)
	SHR-SP	3.5 [#]	(0.9)	2.9 [#]	(0.5)
30W	WKY	2.1	(0.6)	2.8 [#]	(0.9)
	SHR-SP	4.6 [#]	(1.1)	4.5 ⁺	(1.0)

Results are expressed as mean (SD).

There were no significant differences between the apex and base in each group.

[#]: Significant difference in the same portion from the rats of the same strain at 8 weeks. $p < 0.01$

⁺: Significant difference in the same portion from the rats of the same strain at 18 weeks. $p < 0.01$

weeks the percentage areas of perivascular fibrosis of both the small arteries and arterioles in SHRSP were larger than those in WKY. At 30 weeks these differences became larger (Fig. 13). There was no significant difference between the apex and base in the percentage area of perivascular fibrosis of either the small arteries or arterioles in each group, except in that of the arterioles in WKY at 30 weeks (Table III).

DISCUSSION

Myocardial fibrosis in SHRSP increased with advancing age and consisted of focal fibrosis, interstitial fibrosis, and perivascular fibrosis. Myocardial fibrosis in the control WKY tended to increase with advancing age as well, but the fibrosis in SHRSP increased much more progressively. Hypertension is suggested to accelerate the aging process expressed by fibrosis.

Focal fibrosis was seen in older SHRSP and increased with advancing age, although it was not measured in this study. It is supposed that focal fibrosis occurs as a result of injury in partial myocardium, which is supplied by the affected arterioles, because there was a stenosis or an occlusion of arterioles due to thrombosis or thickening of the vessel walls, and because granulomatous focal lesions were often observed in the hearts of 18- and 30-week-old SHRSP. Anderson et al⁷ also said that macroscopic and microscopic scars were almost certainly the result of replacement of irreversibly damaged myocardium.

Ursell et al⁸ are of the opinion that perivascular fibrosis is a response to myocardial ischemia, and that it is a feature of ischemic heart disease. However, there is some question about this view. If perivascular fibrosis were caused by ischemia, it would appear first around the distal arterioles, which are inclined to cause ischemia, rather than around the proximal arteries. In the present study, perivascular fibrosis of small arteries appeared earlier and more prominently than that of arterioles. Besides, there were no stenotic or occlusive changes of vessels at 8 weeks of age. Therefore, the perivascular fibrosis does not appear to be a response to myocardial ischemia. Because there are transient forms between perivascular fibrosis and interstitial fibrosis, both changes seem to be caused by the same etiological factor.

Factor et al observed and analyzed myocardial fibrosis in hypertensive rats and hypertensive-diabetic rats⁹. They concluded that microangiopathy resulting in altered vascular permeability was responsible for interstitial fibrosis.

Byrom¹⁰ suggested that the spasm of cerebral vessels in hypertensive rats caused ischemia, and that consequently increased capillary permeability with attendant focal edema occurred. On the other hand, Giese¹¹ reported that acute hypertension caused constrictions and dilatations of the intestinal arteries of rats, and that the permeability increased in the walls of dilated arterial segments. Hazama et al^{12,13} suggested that necrosis with cyst formation in the brain of SHRSP was a sequela of chronic edema due

Fig.13. The percentage areas of perivascular fibrosis of small arteries and arterioles at different ages.

*: Significant difference between SHRSP and WKY at the same age. $p < 0.01$

**: Significant difference between SHRSP and WKY at the same age. $p < 0.05$

TABLE III PERCENTAGE AREAS OF PERIVASCULAR FIBROSIS OF SMALL ARTERIES AND ARTERIOLES IN THE APEX AND BASE

		Small artery (%)				Arteriole (%)			
		Apex		Base		Apex		Base	
8W	WKY	7.2	(2.1)	5.3	(1.4)	9.8	(3.5)	9.1	(4.0)
	SHR-SP	9.6	(4.2)	10.0	(2.2)	10.9	(8.7)	10.7	(3.9)
18W	WKY	8.4	(2.1)	7.9	(3.7)	9.5	(1.7)	13.4	(5.9)
	SHR-SP	17.2##	(6.2)	23.5##	(11.4)	23.0##	(10.0)	28.2#	(14.5)
30W	WKY	8.4	(3.2)	9.5##	(4.1)	14.6++	(4.7)	11.7	(3.8)
	SHR-SP	25.9++	(6.5)	26.9#	(5.8)	48.5+	(23.3)	33.4***	(9.6)

Results are expressed as mean (SD).

**: Significant difference between the apex and base in either the small arteries or arterioles in each group. $p < 0.05$

: Significant difference in the same portion from the rats of the same strain at 8 weeks. $p < 0.01$

##: Significant difference in the same portion from the rats of the same strain at 8 weeks. $p < 0.05$

+ : Significant difference in the same portion from the rats of the same strain at 18 weeks. $p < 0.01$

++: Significant difference in the same portion from the rats of the same strain at 18 weeks. $p < 0.05$

to an increased permeability of vessels, because the site of lesions corresponded well with the location of increased permeability. In our preliminary study using horseradish peroxidase as a tracer, edema fluid was

observed accumulated particularly in the perivascular regions of small arteries in the hearts of SHRSP. Similar mechanisms seem to be working in the development of interstitial fibrosis as well as perivascular fibrosis

in the hearts of SHRSP. That is to say, the fibrosis seems to be related to increased vascular permeability.

In our preliminary immunohistochemical study on the proliferation in the hearts of SHRSP using bromodeoxyuridine,¹⁴ more labeled nuclei of endothelial cells in small arteries were observed in SHRSP than in WKY in the earlier weeks of age. The labeling of endothelial cells can be regarded as a regenerative reaction to the injury by hypertension, so it is supposed that vascular permeability is elevated by the injury of the cells, and that some permeated growth factors, such as a platelet-derived growth factor,¹⁵ an epidermal growth factor,¹⁶ a macrophage derived growth factor,¹⁷ an endothelial cell-derived growth factor,¹⁸ etc., may cause proliferation of fibroblasts, resulting in increased fibrosis in the hearts of SHRSP.

In the same immunohistochemical study, more labeled nuclei of fibroblasts as well as endothelial cells were observed in small arteries than in arterioles in SHRSP at 8 weeks of age. These findings seem to be concerned with the earlier appearance of perivascular fibrosis of small arteries than that of arterioles.

In SHRSP the degree of myocardial fibrosis seemed to increase from the outer to the inner third of the LVF. It has been reported that wall stress¹⁹ and myocardial fiber diameters²⁰ in hearts with hypertensive heart disease show an increase from the epicardial to the endocardial layers of the LVF. We cannot deny the possibility that wall stress plays a role in the development of myocardial fibrosis in SHRSP.³ Catecholamines might also be involved in the pathogenesis of myocardial fibrosis, because it has been reported that in SHR norepinephrine turnover of the heart increases,²¹ and that isoproterenol, a potent agonist, causes interstitial fibrosis to accompany myocardial hypertrophy.²²

In this study, a color image processor (Luzex 2D, Nikon) was used, which converts RGB (red, green, and blue) signal levels in a color image to elements of intensity, hue, and purity, and displays them as silhouettes. The area of silhouettes was measured. The processor has an excellent ability to discriminate colors and analyze them. It is expected that the measurement of fibrosis by a

color image processor will be used more in the future, because other methods of grading fibrosis are considerably subjective,⁹ and because using a point-count technique is troublesome.^{23,24}

Acknowledgment

Thanks are due to P. Schneider, Shiga University of Medical Science, for assistance in preparing the manuscript.

REFERENCES

1. SCOTTI SM, HACKEL DB: Heart. In Anderson's Pathology, volume one, et by KISSANE JM, The C.V. Mosby Co., St. Louis, Toronto, Princeton, 1985, p560
2. HAZAMA F, OOSHIMA A, TANAKA T, TOMIMOTO K, OKAMOTO K: Vascular lesions in the various substrains of spontaneously hypertensive rats and the effects of chronic salt ingestion. *Jpn Circ J* **39**: 7, 1975
3. TANAKA M, FUJIWARA H, ONODERA T, DER-JINN WU, HAMASHIMA Y, KAWAI C: Quantitative analysis of myocardial fibrosis in normals, hypertensive hearts, and hypertrophic cardiomyopathy. *Br Heart J* **55**: 575, 1987
4. OKAMOTO K, YAMARI Y, NAGAOKA T: Establishment of the stroke-prone spontaneously hypertensive rat (SHR). *Circ Res* **34-35** (Suppl 1): 143, 1974
5. OKAMOTO K, AOKI K: Development of a strain of spontaneously hypertensive rats. *Jpn Circ J* **27**: 282, 1963
6. KURIBAYASHI T: Spontaneously occurring hypertrophic cardiomyopathy in the rat. I. Pathologic features. *Jpn Circ* **51**: 573, 1987
7. ANDERSON KR, SUTTON MSJ, LIE JT: Histopathological types of cardiac fibrosis in myocardial disease. *J Pathol* **128**: 79, 1979
8. URSELL PC, FENOGLIO JJ: Spectrum of cardiac disease diagnosed by endocardial biopsy. *Pathol Annu* **19**(2): 197, 1984
9. FACTOR SM, BAHN R, MINASE T, WOLINSKY H, SONNENBLICK EH: Hypertensive-diabetic cardiomyopathy in the rat; an experimental model of human disease. *Am J Pathol* **102**: 219, 1981
10. BYROM FB: The pathogenesis of hypertensive encephalopathy and its relation to the malignant phase of hypertension. Experimental evidence from the hypertensive rat. *Lancet* **2**: 201, 1954
11. GIESE J: Acute hypertensive vascular disease. Two studies on vascular reaction patterns and permeability changes by means of vital microscopy and colloidal tracer technique. *Acta Pathol Microbiol Scand* **62**: 497, 1964
12. HAZAMA F, AMANO S, HAEBARA H, OKAMOTO K: Changes in vascular permeability in the brain of stroke-prone spontaneously hypertensive rats studied with peroxidase as a tracer. *Acta Pathol Jpn* **25**: 565, 1975
13. HAZAMA F, AMANO S, HAEBARA H,

- YAMORI Y, OKAMOTO K: Pathology and pathogenesis of cerebrovascular lesions in spontaneously hypertensive rats. In *The Cerebral Vessel Wall*, ed by J Cervós-Navarro et al., Raven Press, New York, 1975, p245
14. MORSTYN G, HSU SM, KINSELLA T, GRATZNER H, RUSSO A, MITCHELL JB: Bromodeoxyuridine in tumors and chromosomes detected with a monoclonal antibody. *J Clin Invest* **72**: 1844, 1983
 15. ROSS R, VOGEL A: The platelet-derived growth factor. *Cell* **14**: 203, 1978
 16. OKA Y, ORTH DN: Human plasma epidermal growth factor/ β -urogastrone is associated with blood platelets. *J Clin Invest* **72**: 249, 1983
 17. GLENN KC, ROSS R: Human monocyte-derived growth factor (s) for mesenchymal cells: Activation of secretion by endotoxin and concanavalin A. *Cell* **25**: 603, 1981
 18. GAJDUSEK C, DICORLETO P, ROSS R, SCHWARTZ SM: An endothelial cell-derived growth factor. *J Cell Biol* **85**: 467, 1980
 19. MIRSKY J: Left ventricular stresses in the intact human heart. *Biophys J* **9**: 189, 1969
 20. HOSHINO T, FUJIWARA H, KAWAI C, HAMASHIMA Y: Myocardial fiber diameter and regional distribution in the ventricular wall of normal adult hearts, hypertensive hearts and hearts with hypertrophic cardiomyopathy. *Circulation* **67**: 1109, 1983
 21. YAMORI Y: Contribution of cardiovascular factors to the development of hypertension in spontaneously hypertensive rats. A preliminary report. *Jpn Heart J* **15**: 194, 1974
 22. TANAKA M, TSUCHIHASHI Y, KATSUME H, IJICHI H, IBATA Y: Comparison of cardiac lesions induced in rats by isoproterenol and by repeated stress of restraint and water immersion with special reference to etiology of cardiomyopathy. *Jpn Circ J* **44**: 971, 1980
 23. WEUBEL ER: Stereological principles for morphometry in electron microscopic cytology. *Int Rev Cytol* **26**: 235, 1969
 24. UNVERFERTH DV, BAKER PB, PEARCE LI, LAUTMAN J, ROBERTS WC: Regional myocyte hypertrophy and increased interstitial myocardial fibrosis in hypertrophic cardiomyopathy. *Am J Cardiol* **59**: 932, 1987