Myocardiopathy and Expression of Atrial Natriuretic Peptide in Rats with Monocrotaline-Induced Pulmonary Hypertension

Etsuro HIROKAWA^{1,2)}, Takao KOTANI¹⁾, Jyoji YAMATE¹⁾, Mitsuru KUWAMURA¹⁾, and Sadashige SAKUMA¹⁾

¹⁾Department of Veterinary Pathology, College of Agriculture, University of Osaka Prefecture, 1-1 Gakuen-cho, Sakai, Osaka 593 and

²⁾Research Laboratories, Nippon Shoji, Ltd., 2-24-3 Sho, Ibaraki, Osaka 567, Japan

(Received 22 November 1993/Accepted 18 February 1994)

ABSTRACT. Myocardiopathy in rats with monocrotaline (MCT)-induced pulmonary hypertension was investigated morphologically and immunohistochemically. A single subcutaneous injection of MCT (60 mg/kg body weight) to SD rats produced progressive cardiac lesions. Histologically, the lesions were characterized by myocardial hypertrophy and myocardial degeneration followed by mononuclear cell infiltration and fibroblast proliferation in the right atrium and ventricle. Such histological changes began to be seen 3 weeks after injection and thereafter progressively developed in rats killed 4 and 5 weeks after injection. These findings indicate progressive hypertrophic myocardiopathy, due probably to pulmonary hypertension induced by MCT. Immunohistochemically, atrial natriuretic peptide (ANP)-positive myocardial cells were frequently observed in the left and right ventricle in MCT-treated rats killed 4 and 5 weeks after injection. The intensive immunopositive reaction was observed mainly in hypertrophic myocardial cells in the subendocardium of the right ventricle and also present in hypertrophic myocardial cells around injured areas consisting of degenerated myocardial cells, mononuclear cell infiltration and fibrosis. These findings suggest a close relationship between the ANP expression and cardiac hypertrophy in MCT-treated rats.—**KEY WORDS:** atrial natriuretic peptide, monocrotaline, myocardiopathy, pulmonary hypertension, rat.

J. Vet. Med. Sci. 56(4): 651–655, 1994

Monocrotaline (MCT), a kind of pyrrolizidine alkaloid isolated from *Crotalaria spectabilis* seeds, is converted to active pyrroles by the complex oxidase systems including cytochrome P-450 in the liver when taken into the body. The pyrroles are considered to cause cardiopneumotoxicity [12]. Morphological studies on pulmonary vascular changes developing progressively after a single injection of MCT have been reported in detail in rats [1, 10, 14]. The vascular changes were characterized histologically by perivascular edema, wall thickness and cell infiltration [9, 14]. Although changes in the myocardium have been described in MCT-treated rats [4, 9], no chronological histology has been given to MCT-induced myocardiopathy in rats.

Atrial natriuretic peptide (ANP) is a circulating hormone which is synthesized and secreted predominantly by atrial myocardium in healthy adult mammals [2, 6]. The ANP has been considered to regulate body fluids, electrolytes and vascular homeostasis [2]. It has been recently reported in humans [8, 15, 16], hamsters [3] and dogs [5] that ANP-immunopositive cells were seen in ventricular myocardial cells in some pathological conditions such as dilated or hypertrophic myocardiopathy and hypertensive heart disease, suggesting a close relationship between the overexpression of ANP and cardiac disorders.

In the present study, we investigated the relationship between the expression of ANP and changes in the myocardium in MCT-treated rats.

MATERIALS AND METHODS

Animals: A total of forty 6-week-old Crj/CD (SD) male rats, Charles River Japan, Inc., were used. They were

housed at a temperature of $22 \pm 4^\circ\text{C}$ and given commercial laboratory diet (MF, Oriental Yeast Co., Ltd., Osaka, Japan) and water *ad libitum*.

MCT injection: Two % of MCT solution was prepared according to the methods of Hayashi *et al.* [10]. Briefly, 200 mg of crystalline MCT (Trans World Chemicals, Washington, U.S.A.) were dissolved in 1.2 ml of 1N HCl solution, and the acidified solution was diluted to about 5 ml with distilled water. After neutralization with 0.5N NaOH, the volume was adjusted to 10 ml. Twenty rats were once injected subcutaneously with the MCT solution at a dose of 60 mg/kg body weight, and the remaining twenty rats were once injected with physiological saline and served as controls. During the observation period for 5 weeks, all rats used were weighed twice weekly. Four rats of each group were killed under ether anesthesia 1, 2, 3, 4 and 5 weeks after the injection, respectively.

Histopathology: Heart of each animal was cut into three parts (one longitudinal and two half transverse sections including the right ventricle), and fixed in Zamboni's solution after being weighed. Lungs were fixed in 10% neutral buffered formalin. After the fixation, tissues were embedded in paraffin. Sections from the heart were stained with hematoxylin and eosin and phosphotungstic acid-hematoxylin, and sections from the lung were stained with victoria blue-hematoxylin and eosin. In order to evaluate hypertrophied myocardial cells, the diameter of randomly-selected fifty myocardial cells that directly crosses the center of the nucleus in longitudinal section of the right ventricle was measured by using a micrometer.

Immunohistochemistry: For the immunohistochemical demonstration of ANP, an avidin-biotin complex (ABC) immunoperoxidase technique was carried out. After

blocking endogenous peroxidase by treatment with 0.3% H₂O₂ in methanol for 20 min, deparaffinized sections were treated with 10% normal goat serum in phosphate buffered saline (PBS) for 20 min. A rabbit polyclonal ANP (human, 1–28) antiserum (Peptide Institute, Osaka, Japan) at a dilution of 1:2000 was applied to sections overnight at 4°C. After rinsing in PBS, the sections were incubated with a biotinylated goat antirabbit immunoglobulin (DAKO, Denmark) diluted 1:400 for 1 hr at room temperature. Subsequently, streptavidin-biotin-peroxidase complex (DAKO) was reacted to the sections for 1 hr at room temperature. The sections were then stained with 3,3'-diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries, Osaka, Japan) for 5 min, and counterstained with Mayer's hematoxylin. PBS or normal rabbit serum was used as negative control instead of the primary antibody.

Statistical analysis: Values of body weight, heart weight as well as the myocardial cells in diameter were compared between the control and MCT-treated group by Student's unpaired-*t* test. Statistic significance was at $P < 0.05$.

RESULTS

Clinical outcome: All MCT-treated rats showed progressive tachypnea and dyspnea from 3 weeks after injection. The body weight of MCT-treated group was significantly lower than that of the control group from the first week (Table 1).

Macroscopic findings: Slight hydrothorax was observed in all MCT-treated rats killed 2 and 3 weeks after injection. In addition to the hydrothorax, discoloration and collapse of the lungs were seen in all MCT-treated rats killed 4 and 5 weeks after injection. In all MCT-treated rats killed 4 and 5 weeks after injection, right atrial and ventricular dilation and right ventricular hypertrophy were observed, and one of these rats showed bisheart.

In the MCT-treated group, absolute heart weight after 4 and 5 weeks and relative heart weight after 3, 4 and 5

weeks significantly increased compared with those in the control group, respectively (Table 1).

Histopathological findings: The vascular changes observed in the lungs of all MCT-treated rats were similar to those reported previously [10]. After 1 and 2 weeks, the changes appeared in some pulmonary arterioles and venules, and were composed of perivascular edema and infiltration of a small number of mononuclear cells. Besides, aggregations of foamy macrophages were present in some alveoli. After 3 weeks, many pulmonary arterioles showed wall thickness due to hypertrophic medial smooth muscle cells. After 4 and 5 weeks, wall thickness of pulmonary arterioles became more prominent, accompanying thrombus formation.

No noticeable changes were detected in the heart of MCT-treated rats killed 1 and 2 weeks after injection. Focal lesions consisting of a few degenerating myocardial cells, infiltration of a few mononuclear cells and proliferation of fibroblasts were observed in the right ventricle in two of four MCT-treated rats killed after 3 weeks. After 4 and 5 weeks, in all MCT-treated rats, hypertrophic and/or degenerating myocardial cells, infiltration of mononuclear cells around injured areas and fibrosis were seen in the right atrium, ventricle and right side of the septum (Figs. 1 and 2). The lesions were multifocal or diffuse; the myocardial hypertrophy was diffusely seen, whereas myocardial degeneration accompanied by mononuclear cell infiltration and fibrosis was multifocally observed in the inner two-thirds of the right ventricle. These lesions was more severe after 5 weeks. The degenerated myocardial cells were characterized by cytoplasmic hyalinization and/or vacuolation, disappearance of longitudinal striations and sarcoplasmic disruption. In addition, eosinophilic granules probably derived from abnormal accumulation of myofilaments were occasionally seen in their cytoplasm. There were no noticeable changes in intramural coronary arteries in MCT-treated rats.

After 4 and 5 weeks, diameter of myocardial cells in the right ventricle of MCT-treated rats significantly increased

Table 1. Mean body weight, absolute and relative heart weight, and size of myocardial cells in control and monocrotaline (MCT)-treated rats

Item	Group ^{a)}	Weeks after injection				
		1	2	3	4	5
Mean body weight (g)	Control	266.4 ± 8.8 ^{b)}	317.0 ± 12.0	349.2 ± 13.7	384.0 ± 23.8	390.8 ± 28.7
	MCT	244.7 ± 7.2**	288.3 ± 12.0**	321.9 ± 17.8**	307.4 ± 38.9**	277.3 ± 41.2**
Absolute heart weight (g)	Control	1.001 ± 0.119	1.094 ± 0.076	1.132 ± 0.064	1.229 ± 0.035	1.234 ± 0.082
	MCT	0.908 ± 0.113	1.027 ± 0.086	1.194 ± 0.076	1.460 ± 0.130*	1.499 ± 0.183*
Relative heart weight (%)	Control	0.385 ± 0.029	0.332 ± 0.019	0.331 ± 0.059	0.312 ± 0.017	0.317 ± 0.030
	MCT	0.368 ± 0.032	0.353 ± 0.018	0.356 ± 0.018*	0.498 ± 0.064**	0.543 ± 0.041**
Size of myocardial cell (μm)	Control	17.4 ± 1.3	19.9 ± 1.2	20.0 ± 2.6	20.7 ± 0.8	20.4 ± 1.2
	MCT	18.6 ± 1.5	21.4 ± 2.4	22.5 ± 1.2	30.1 ± 3.2**	35.1 ± 1.2**

a) Four rats each in the control and MCT-treated groups were examined. b) Mean ± standard deviation. Significantly different from control; * $P < 0.05$, ** $P < 0.01$.

Fig. 1. Longitudinal section of right ventricle in a monocrotaline (MCT)-treated rat killed 4 weeks after injection showing myocardial hypertrophy, mononuclear cell infiltration and interstitial fibrosis. Hematoxylin and eosin (HE) stain. $\times 350$.

Fig. 2. Cross section of right ventricle in MCT-treated rat killed 4 weeks after injection showing myocardial hypertrophy and interstitial fibrosis. Myocardial cells with vacuolated cytoplasm are also seen. HE stain. $\times 350$.

Fig. 3. Atrial natriuretic peptide (ANP)-immunopositive myocardial cells seen in the right auricle in control (A) and MCT-treated (B) rat killed 4 weeks after injection. In contrast to control rat, the intensity of the immunoreaction reduces in MCT-treated rat. Counterstained with hematoxylin. $\times 280$.

Fig. 4. ANP-immunopositive myocardial cells (arrows) seen in the subendocardium of the right ventricle in MCT-treated rat killed 5 weeks after injection. Counterstained with hematoxylin. $\times 280$.

as compared with that in control, respectively (Table 1).

Immunohistochemical findings: The ANP-positive granules tended to concentrate around the nuclei of myocardial cells. In some cells, however, the positive granules were located in the subsarcolemma.

ANP-positive myocardial cells were distributed in the left and right auricles and the left subendocardium of the septum of all of the control and MCT-treated rats. However, the intensity for ANP-immunopositivity tended to reduce in the right auricle of all MCT-treated rats killed 4 and 5 weeks after injection, in contrast to control rats (Fig. 3). In two of eight MCT-treated rats killed 2 and 3 weeks after injection, a small number of the positive cells were seen in the left subendocardium of the apex. After 4 and 5 weeks, the positive cells were seen diffusely throughout the right and left ventricles in all MCT-treated rats (Fig. 4); particularly, the strong immunopositivity for ANP was observed in hypertrophic myocardial cells in the subendocardium of the right ventricle and in those around injured areas which consisted of degenerated myocardial cells, infiltration of mononuclear cells and fibrosis (Fig. 5). Distribution of ANP-positive myocardial cells in the heart of MCT-treated rats killed at 4 and 5 weeks is summarized in Fig. 6.

DISCUSSION

It has been shown in rats received a single dose of MCT that the pulmonary arterial pressure (PAP) gradually

Fig. 5. Right ventricle in MCT-treated rat killed 4 weeks after injection showing ANP-immunopositive myocardial cells (arrows) seen around injured area, consisting of degeneration of myocardial cells and interstitial fibrosis. Counterstained with hematoxylin. $\times 280$.

Fig. 6. Distribution of ANP-immunopositive myocardial cells in the heart of MCT-treated rat killed 4 and 5 weeks after injection. The dot represents areas including various degree of positive myocardial cells. The immunopositive reaction is characteristically observed in the right and left ventricles of MCT-treated rats. RA, right auricle; RV, right ventricle; SP, septum; LA, left auricle; LV, left ventricle; A, aorta; PA, pulmonary artery.

elevated with time and the ratio of right ventricular weight to left ventricle plus septum weight ($RV/LV+S$) and medial thickness of the small pulmonary arteries increased in proportion to the elevated PAP [7]. Pulmonary vascular changes observed in the present study have been reported to be characteristic in rats with MCT-induced pulmonary hypertension [7,10]. This indicated the presence of pulmonary hypertension in our MCT-treated rats. It was considered that the persistent pulmonary hypertension might cause myocardial injury in the right ventricle, resulting from overload by more forceful contraction. Therefore, the myocardial degeneration with reactive cell infiltration as well as fibroblast proliferation, which were observed in the right ventricle of MCT-treated rats killed 3 weeks after injection, was considered to be an early stage of myocardial injury which developed in association with elevated blood pressure due to pulmonary hypertension. A significant increase in relative heart weight after 3 weeks in MCT-treated rats might reflect such cardiac lesions.

The present study further demonstrated the presence of more advanced cardiac lesions in MCT-treated rats killed 4 and 5 weeks after injection. The lesions might be regarded as the progressive hypertrophic cardiomyopathy. Right ventricular hypertrophy was considered to be a

result of continued mechanical stimulus caused by hemodynamic overload provoked in association with pulmonary hypertension [7]. Furthermore, the myocardial degeneration and fibrosis might develop diastolic dysfunction of the right ventricle, resulting in relative cardiac decompensation. Mononuclear cells observed in cardiac lesions of MCT-treated rats particularly killed 4 and 5 weeks after injection were regarded as infiltrating macrophages, because most of these cells were immunoreactive for rat monocyte/macrophage-specific antibody (ED-1: unpublished data). Therefore, the cell infiltration should be considered to be a reactive change to degenerated myocardial cells.

Mckenzie *et al.* [13] reported in rats with experimental pulmonary hypertension induced by chronic hypoxia that a decrease in ANP concentration in the right atrium was detected by specific radioimmunoassay. The reduction of ANP in the right auricle in this study may result from the continuous secretion of ANP by chronic stretch of the atrial myocardium [13].

The abnormal appearance of ANP-positive cells in MCT-treated rats might be caused by pressure overload to the right ventricle due to pulmonary hypertension. In agreement with our findings, Hirata *et al.* [11] have confirmed overexpression of ANP-specific mRNA in MCT-induced rat cardiac hypertrophy by using Northern blot analysis. ANP has been well known to decrease blood pressure through natriuretic effects and vasodilation [2, 6]. Therefore, the increased number of ANP-positive cells in the right ventricle was considered to be adaptation to abnormal hemodynamic overload; it was regarded as a marker for the presence of myocardial hypertrophy. Since no histologically noticeable changes were detected in the left ventricle, significance of appearance of ANP-positive myocardial cells in the left ventricle remains unclear. The intensive immunopositive reaction for ANP, which were frequently observed in hypertrophic myocardial cells around degenerated myocardial cells in the right subendocardium of the septum, might also be a result of response to regional myocardial changes. Because, Takemura *et al.* [15] reported that ANP-immunopositive cells appeared exclusively around degenerated myocardial cells in the septum of human patients with hypertrophic cardiomyopathy, and speculated that expression of ANP might have been induced by regional myocardial injury.

In conclusion, the present study revealed that myocardial hypertrophy and myocardial degeneration followed by mononuclear cell infiltration and interstitial fibrosis progressively developed mostly in the right ventricle of MCT-treated rats. Furthermore, we showed that ANP-immunopositive cells abnormally appeared with the advancing cardiac lesions, suggesting a close relationship between the ANP expression and cardiac hypertrophy in MCT-treated rats.

REFERENCES

- Butler, W. H. 1970. An ultrastructural study of the pulmonary lesion induced by pyrrole derivatives of the pyrrolizidine alkaloids. *J. Pathol.* 102: 15-19.
- Cantin, M. and Genest, J. 1985. The heart and the atrial natriuretic factor. *Endocrine Rev.* 6: 107-127.
- Cantin, M., Thibault, G., Ding, J., Gutkowska, J., Garcia, R., Jasmin, G., Hamet, P., and Genest, J. 1988. ANF in experimental congestive heart failure. *Am. J. Pathol.* 130: 552-568.
- Chesney, C. F., Allen, J. R., and Hsu, I. C. 1974. Right ventricular hypertrophy in monocrotaline pyrrole treated rats. *Exp. Mol. Pathol.* 20: 257-268.
- Colbatzky, F., Vollmar, A., Mönch, U., and Hermanns, W. 1993. Synthesis and distribution of atrial natriuretic peptide (ANP) in hearts from normal dogs and those with cardiac abnormalities. *J. Comp. Pathol.* 108: 149-163.
- De Bold, A. J., Borenstein, H. B., Veress, A. T., and Sonnenberg, H. 1981. A rapid and potent natriuretic response to intravenous injection of atrial myocardial extract in rats. *Life Sci.* 28: 89-94.
- Ghodsi, F. and Will, J. A. 1981. Changes in pulmonary structure and function induced by monocrotaline intoxication. *Am. J. Physiol.* 240: H149-H155.
- Hasegawa, K., Fujiwara, H., Doyama, K., Mukoyama, M., Nakao, K., Fujiwara, T., Imura, H., and Kawai, C. 1993. Ventricular expression of atrial and brain natriuretic peptides in dilated cardiomyopathy. *Am. J. Pathol.* 142: 107-116.
- Hayashi, Y., Hussa, J. F., and Lalich, J. J. 1967. Cor pulmonale in rats. *Lab. Invest.* 16: 875-881.
- Hayashi, Y., Kokubo, T., Takahashi, M., Furukawa, F., Otsuka, H., and Hashimoto, K. 1984. Correlative morphological and biochemical studies on monocrotaline-induced pulmonary alterations in rats. *Toxicol. Lett.* 21: 65-71.
- Hirata, Y., Suzuki, E., Hayakawa, H., Matsuoka, H., Sugimoto, T., Kojima, M., Kangawa, K., and Matsuo, H. 1992. Role of endogenous ANP in sodium excretion in rats with experimental pulmonary hypertension. *Am. J. Physiol.* 262: H1684-H1689.
- Mattocks, A. R. 1968. Toxicity of pyrrolizidine alkaloids. *Nature (Lond.)* 217: 723-728.
- Mckenzie, J. C., Tanaka, I., Inagami, T., Misono, K. S., and Klein, R. M. 1986. Alterations in atrial and plasma atrial natriuretic factor (ANF) content during development of hypoxia-induced pulmonary hypertension in the rat. *Proc. Soc. Exp. Biol. Med.* 181: 459-463.
- Reindel, J. F., Ganey, P. E., Wagner, J. G., Slocombe, R. F., and Roth, R. A. 1990. Development of morphologic, hemodynamic and biochemical changes in lungs of rats given monocrotaline pyrroles. *Toxicol. Appl. Pharmacol.* 106: 179-200.
- Takemura, G., Fujiwara, H., Mukoyama, M., Saito, Y., Nakao, K., Kawamura, A., Ishida, M., Kida, M., Uegaito, T., Tanaka, M., Matsumori, A., Fujiwara, T., Imura, H., and Kawai, C. 1991. Expression and distribution of atrial natriuretic peptide in human hypertrophic ventricle of hypertensive hearts and hearts with hypertrophic cardiomyopathy. *Circulation* 83: 181-190.
- Takemura, G., Fujiwara, H., Yoshida, H., Mukoyama, M., Saito, Y., Nakao, K., Fujiwara, T., Uegaito, T., Imura, H., and Kawai, C. 1990. Identification and distribution of atrial natriuretic polypeptide in ventricular myocardium of humans with myocardial infarction. *J. Pathol.* 161: 285-292.