ORIGINAL

Effects of cardiotrophin-1 on hemodynamics and cardiomyocyte apoptosis in rats with acute myocardial infarction

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Abstract : The effects of cardiotrophin-1 on hemodynamics, cardiac function, cardiomyocyte apoptosis, and expression of P53, Fas, Bax and Bcl-2 proteins in myocardium were determined in a rat model of acute myocardial infarction. Twenty-four male Sprague-Dawley rats weighing approximately 310 g were subjected to left coronary artery ligation. Seven days before surgery, the rats were randomized to receive cardiotrophin-1 (treated group) or phosphate-buffered saline (control group). Recombinant rat cardiotrophin-1 (2 μg in 1 ml phosphate-buffered saline) or phosphate-buffered saline (1ml) was administered daily via the tail vein for 7 days (n=12 for each group). Hemodynamic parameters, apoptotic index, P53, Fas, Bax and Bcl-2 expression in myocardium were measured at 24 hours after coronary ligation. As compared with control animals, rats treated with cardiotrophin-1 had significantly higher mean arterial pressure, left ventricular systolic pressure and the maximum rate of left ventricular pressure rise or fall, and significantly lower left ventricular end-diastolic pressure. Cardiotrophin-1 pretreatment did not affect the heart rate, heart weight, body weight or the ratio of heart weight to body weight. The number of apoptotic cardiomyocytes in cardiotrophin-1 treated group was less than that in control group [(15.8±5.2) % *vs* (34.6±7.7) %, P<0.01]. Cardiotrophin-1 pretreatment significantly inhibited P53, Fas and Bax, and increased Bcl-2 expression in myocardium. J. Med. Invest. 51 : 29-37, February, 2004

Keywords **:** *myocardial infarction, cardiotrophin-1, apoptosis*

INTRODUCTION

The common view on how cardiomyocytes die during or after myocardial infarction has altered in recent years. For a long time necrosis was regarded as the sole cause of cell death in myocardial infarction. Now, recent studies indicate that apoptosis also plays a role in the process of tissue damage subsequent to myocardial infarction (1-4). Cardiotrophin-1 (CT-1) is a new member of the interleukin (IL)-6 family of cytokines. A 1.7 kb CT-1 mRNA is expressed in the human heart and several other tissues. CT-1 shares the signal transducing receptor components gp 130 and leukemia inhibitory factor receptor (LIFR) with the previously identified members of the IL-6 cytokine family. CT-1 is expressed at high levels in the myocardium during the course of cardiogenesis, and promotes the proliferation and survival of embryonic cardiomyocytes. CT-1 may therefore play an important role in several cardiovascular diseases with cardiac hypertrophy as well as heart failure (5-7). More recently, several investigations have demonstrated that CT-1 could effectively reduce myocardial ischemia/reperfusion injury (8 -11) and prevent cardiomyocyte apoptosis (12) *in vitro*. However, whether CT-1 plays an important role in postischemic myocardial apoptosis *in vivo* has not been determined. Therefore, the present study was undertaken to determine the effects of CT-1 on hemodynamics, cardiac function, cardiomyocyte apop-

Received for publication July 17, 2003 ; accepted September 30, 2003.

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tosis, as well as expression of P53, Fas, Bax and Bcl-2 proteins in a rat model of acute myocardial infarction.

MATERIAL AND METHODS

1)Rat infarct model

Twenty-four male Sprague-Dawley rats weighing approximately 310 g were used for this study. The rats were subjected to left anterior descending coronary artery ligation which produced extensive acute myocardial infarction (about 35% of left ventricle) as our previous reports (13, 14), with 12 rats for each group. The animals were anesthetized by intraperitoneal injection of ketamine hydrochloride (80mg/kg), intubated via tracheotomy, and mechanically ventilated with a volume-cycled small-animal respirator. Anterolateral thoracotomy was performed, and the heart was rapidly exteriorized. One or two 5-0 silk sutures were snared in a blinded manner around the proximal left anterior descending coronary artery and then tightly ligated to occlude the vessel. The heart was then replaced, the lungs were reexpanded, and the chest was closed in three layers with 4-0 silk sutures. The animals were kept under mechanical ventilation until they awoke from anesthesia. The study protocol was approved by the Administrative Panel on Laboratory Animal Care of Guangxi Medical University, China.

2)Administration of CT-1

Seven days before surgery, the rats were randomized to receive CT-1(treated group) or phosphate-buffered saline (PBS, control group). 2μg recombinant rat CT-1 (Prepro Tech EC Ltd., London, UK) in 1 ml PBS was injected daily via the tail vein for 7 days. Control rats received an equal volume of PBS alone (n=12 for each group).

3)Hemodynamic monitoring

Twenty-four hours after the surgery, the animals were again anesthetized. A longitudinal incision was then performed in the right cervical part. Through this incision, the right internal carotid artery was isolated and controlled using vessel loops. A polyethylene-50 catheter filled with heparin and saline (200IU/ml) was then inserted through the right internal carotid artery into the left ventricular cavity and connected to a pressure transducer. Left ventricular pressure was continually recorded on a Power Macintosh computer via a data acquisition system. The left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), the maximum rate of left ventricular pressure rise or fall ($\pm dP/dt_{max}$), and heart rate (HR) were automatically analyzed. Mean arterial pressure (MAP) was calculated from the carotid arterial pressure recorded in the right internal carotid artery. At the completion of physiologic measurements, the chest was opened and the heart excised. The atria were separated, the blood in the ventricular chambers removed, the left ventricle inclusive of the septum and right ventricle dissected, and their weights collected. The left ventricle was cut into four transverse slices from apex to base. The slices were fixed at 24 hours in 10% formalin, embedded in paraffin, and cut into 4 μm sections.

4)Terminal deoxynucleotidyl transferase-mediated dUTP-Biotin in situ nick-end labelling (TUNEL)

Sections were mounted on poly-L-lysine-coated slides. After deparaffinization and rehydration, tissue sections were incubated with proteinase K (20 μg/ml in 10 mM Tris-HCl, pH 7.6) for 30 minutes at 21 to 37°C. Sections were rinsed twice with PBS and incubated with blocking solution (0.3% H_2O_2 in methanol) for 1 hour at room temperature. DNA strand breaks were detected using the terminal deoxynucleotidyl transferase mediated dUTP nick end labelling method (TUNEL). The reagents were all from Roche Diagnostics (Hong Kong) Limited. Briefly, sections were covered with 50 μl of TUNEL reaction mixture, and incubated in this solution for 60 minutes at 37°C in a humidified chamber. After rinsing in PBS, the sections can be analyzed under a fluorescence microscope. Subsequently, the samples were covered with 50 μl of converter-POD (anti-fluorescein antibody, Fab fragment from sheep, conjugated with horse-radish peroxidase), and incubated in a humidified chamber for 30 minutes at 37°C. Sections were rinsed three times with PBS. Finally, sections were covered with AEC-substrate solution (50 to 100 μl), and incubated for 10 minutes at room temperature. Sections were rinsed three times with PBS again. The sections were faintly counterstained with hematoxylin before coverslipping with AEC-mounting solusion. The number of apoptotic cardiomyocytes and their percentage of total cardiomyocytes were counted with the use of a microscope. Cardiomyocytes from at least 3 sections per animal that were randomly selected were evaluated immunohistochemically to determine the number and percentage of cells exhibiting positive staining for apoptosis. For each slide 5 fields were randomly chosen, and by using a defined rectangular field area (×40 objective), a total of 200 cells per field were counted. The index of apoptosis was determined (ie, number of apoptotic cardiomyocytes divided by the total number of cardiomyocytes counted×100)

Table 1. Comparisons of hemodynamic characteristics and heart weight between control and treated groups (mean ±SD).

	Control group	Treated group
HR(beats/min)	385.38±12.74	378.32±13.45
MAP(mmHg)	91.62±6.83	103.77±7.63*
LVSP(mmHg)	114.26±7.74	125.88±6.43*
LVEDP(mmHg)	14.79±3.85	9.33±2.68*
$+dP/dt_{max}$(mmHg/s)	4489.76±353.79	5412.28±362.52**
$-dP/dt_{max}$(mmHg/s)	3428.57±321.48	4131.18±286.44**
HW(mg)	1041.55±49.21	1054.33±51.86
BW(g)	308.26±15.47	311.66±14.87
HW/BW(mg/g)	3.38±0.24	3.39±0.21

HR=heart rate ; MAP=mean arterial pressure ; LVSP=left ventricular systolic pressure ; LVEDP=left ventricular end-diastolic pressure ; $\pm dP/dt_{max}$=the maximum rate of left ventricular pressure rise ($+dP/dt_{max}$) or fall ($-dP/dt_{max}$) ; HW=heart weight ; BW=body weight ; HW/BW= the ratio of heart weight to body weight ; *P <0.01,**P <0.001 in comparison with control group.

from a total of 15 fields per heart, and the assays were performed in a blinded manner.

5)Immunohistochemical staining of P53, Fas, Bax, and Bcl-2 proteins in myocardium

Immunohistochemical staining for P53, Fas, Bax, and Bcl-2 proteins was performed on three cross-sections serially cut at 4-μm intervals from each animal. Briefly, this involved blocking endogenous peroxidase with 3% hydrogen peroxide, preincubation in blocking serum, and application of the primary antibody (Santa Cruz Biotechnology, Inc.) at the appropriate dilution (ie, 1 : 80) overnight at 4°C. A biotinylated secondary antibody was then applied for 30 minutes at room temperature followed by streptavidin-horseradish-peroxidase complex. After 30 minutes at room temperature, sections rinsed with PBS and visualized by incubation with 0.05% (w/v) 3, 3'-diaminobenzidine tetrahydrochloride dehydrate. Slides were counterstained with hematoxylin before coverslipping. The value of optical density is monitored with computer-assisted image analysis (LEICA Q 550 IW). For each slide 5 fields were randomly chosen. The value of mean optical density was calculated. The assays were performed in a blinded manner.

6)Statistical analysis

All values in the text and tables are presented as mean±SD. Comparisons between two groups were made using unpaired Student's t test. A value of P< 0.05 was considered to have statistical significance.

RESULTS

1)Effect of CT-1 on hemodynamics

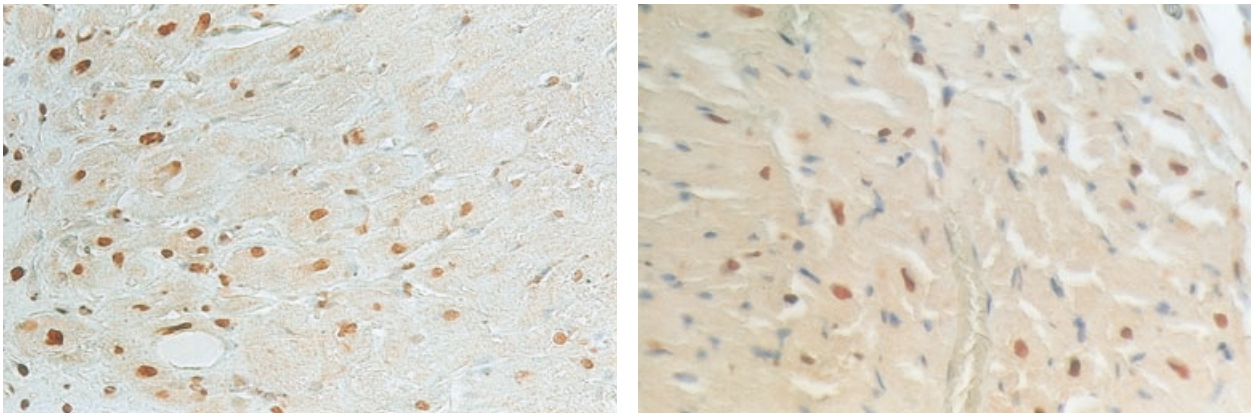
The MAP, LVSP and ± dP/dt_{max} in CT-1 treated group were significantly higher than those in control group, respectively. On the contrary, the LVEDP in treated group was significantly lower than that in control group. CT-1 pretreatment did not affect the HR, heart weight, body weight or the ratio of heart weight to body weight (Table 1).

2)Effect of CT-1 on cardiomyocyte apoptosis

TUNEL staining method showed that many apoptotic cardiomyocytes were observed in the border zone of infarcted tissue in both control and treated animals. The nuclei of the apoptotic cardiomyocytes were red of fluorescence labelling. The amount of apoptotic cardiomyocytes in CT-1 treated group was significantly less than that in control group [(15.8±5.2)% *vs* (34.6±7.7)%, P <0.01](Figure 1).

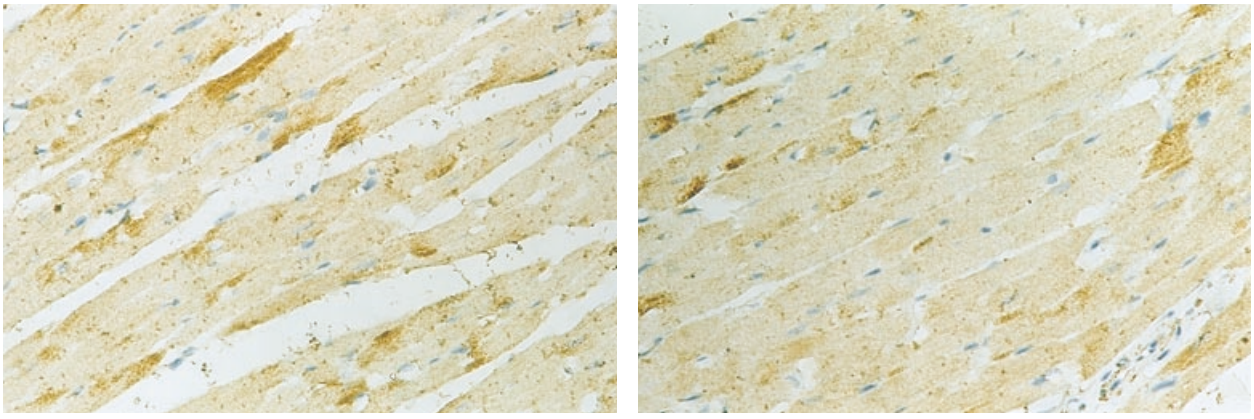
3)Effect of CT-1 on expression of myocardial P53, Fas, Bax and Bcl-2

Immunohistochemical staining showed that P53 immunoreactivity was found in the tissue adjacent to infarcted zone in both control and treated groups. The intensity of P53 protein expression was coincident with the number of cardiomyocyte apoptosis. P53 immunoreactive product was mainly located in the nuclei. The mean optical density in treated group was lower than that in control group (Table 2 and Figure 2) ; Fas immunoreactivity was yellow-brown product. The immunoreactive product was located in the membrane and cytoplasm of cardiomyocytes. The mean optical density of Fas protein in treated group was lower than that in control group (Figure 3) ; Bax immunoreactivity was yellow-brown reactive product which located in the cytoplasm of cardiomyocytes. The mean optical density of Bax protein in treated group was also lower than that in control group (Figure 4) ; Bcl-2 labelling in cardiomyocytes was distributed in the cytoplasm.



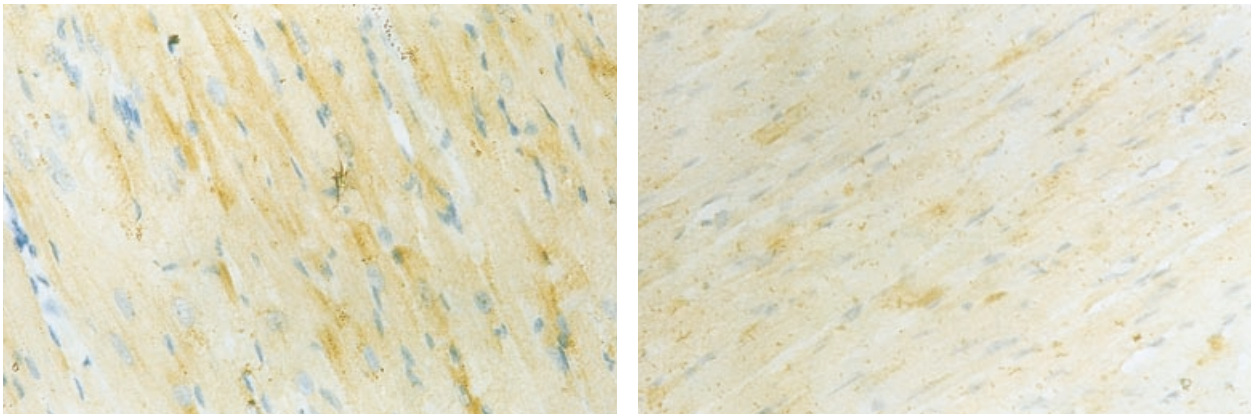
A **B**

Figure 1. Myocardial sections of control (A) and treated (B) animals, the number of apoptotic myocytes (with intensely red nuclei) determined with terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick end labelling (TUNEL) method is more in control section than that in treated section. TUNEL method×400



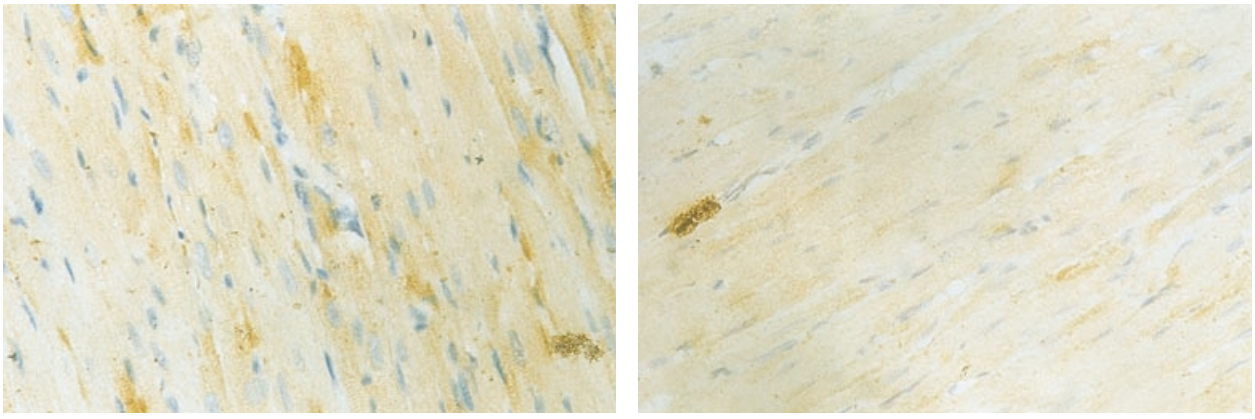
A **B**

Figure 2. Immunohistochemical (IHC) staining for P53 of myocardial sections of control (A) and treated (B) animals, P53 immunoreactivity is stronger in control section than that in treated section. P53 immunoreactive substance was mainly located in the nuclei. IHC method×400



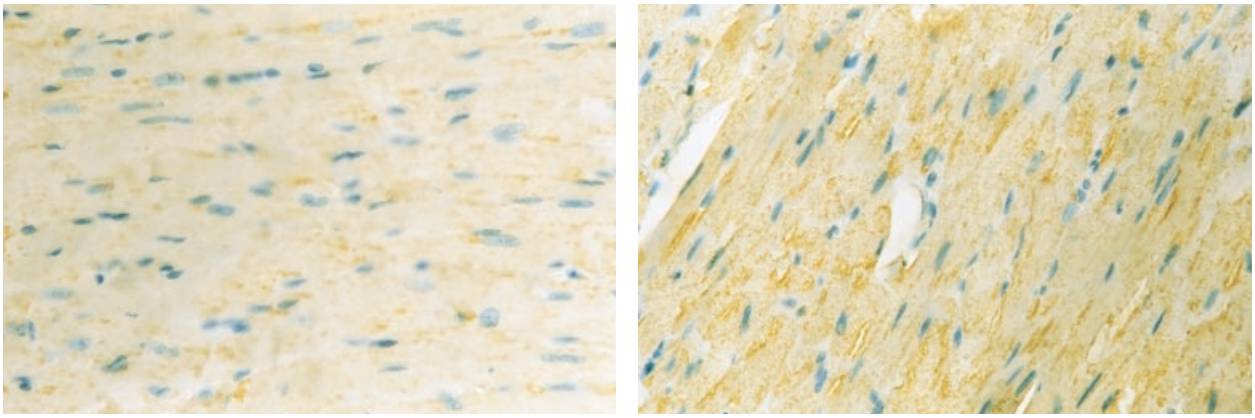
A **B**

Figure 3. Immunostaining for Fas of myocardial sections of control (A) and treated (B) rats, Fas immunoreactivity is stronger in control section than that in treated section. Fas immunoreactive substance was located in the cytoplasm. IHC method×400



A B

Figure 4. Immunohistochemically stained views for Bax of myocardial sections of control (A) and treated (B) rats, Bax immunoreaction is stronger in control section than that in treated section. The yellow-brown reactive product was distributed in the cytoplasm. IHC method×400



A B

Figure 5. Immunostaining for Bcl-2 of myocardial section of control (A) and treated (B) rats, Bcl-2 immunoreactivity is stronger in treated section than that in control section. The immunoreactive substance was mainly distributed in the cytoplasm. IHC method×400

Table 2. Comparisons of mean optical density of myocardial P53, Fas, Bax and Bcl-2 proteins between control and treated groups (mean±SD).

	Control group	Treated group
P53	0.12±0.02	0.07±0.02*
Fas	0.14±0.03	0.09±0.03*
Bax	0.15±0.04	0.08±0.03**
Bcl-2	0.13±0.02	0.18±0.04*

*P <0.05,**P <0.01 in comparison with control group.

The mean optical density of Bcl-2 protein in treated group was significantly higher than that in control group (Figure 5).

DISCUSSION

The chronic effects of CT-1 on hemodynamics and cardiac function are still unclear. Jin *et al*.(15) have reported that intravenous administration of CT-1 caused a dose-dependent decrease in MAP, and an increase in HR. CT-1 (100μg/kg) significantly elevated cardiac output and HR, and decreased MAP and systemic vascular resistance. Stroke volume was unaltered. There was no significant difference in left ventricular maximal dP/dt between the CT-1-treated and vehicle-

treated groups. Yao *et al*. (16) showed that CT-1 injection significantly decreased blood pressure and significantly increased HR in spontaneously hypertensive rats and Wistar Kyoto rats. The most obvious change occurred within 10 minutes after injection. However, there was no significant difference in the hypotensive effect of CT-1 on 10-week-old spontaneously hypertensive rats and Wistar Kyoto rats. Hamanaka *et al*. (17) have also demonstrated that intravenous injection of CT-1(4-100μg/kg) in conscious rats evoked significant declines in blood pressure and reflex increases in HR in a dose-dependent manner. CT-1 induced no significant change in cardiac output, which was compatible with the results from isolated perfused rat hearts ; HR, change in pressure over time, left ventricular developed pressure, and perfusion pressure were unaffected. However, in the present study we showed that pretreatment with CT-1 for 7 days is capable of significantly increasing MAP, LVSP and $\pm dP/dt_{max}$, and significantly lowering LVEDP. CT-1 pretreatment did not affect the HR, heart weight, body weight or the ratio of heart weight to body weight in the rat model of acute myocardial infarction. The reason for this discrepancy may be related to the dosage and course of CT-1 administration. In our current study, the dose of CT-1 is relative small, furthermore, the hemodynamic parameters were measured at 24 hours after the last injection of CT-1, thus the results of the present study may be thought to be the chronic effects of CT-1 on hemodynamics and cardiac function.

Myocardial infarction has been considered to be a prime example of necrotic cell death, because of the breakdown of cellular energy metabolism. However, apoptosis of cardiomyocytes also occurs in a temporally and spatially specific manner(1-4). Thus, acute myocardial infarction manifests both forms of cell death, with apoptosis particularly occurring at the hypoperfused" border "zones, between a central area of necrosis and viable myocardium. The central, unperfused region also manifests apoptosis, particularly within the first six hours, although between 6 to 24 hours necrosis is more common. Apoptosis in the remote non-infarcted myocardium may be partly responsible for myocardial remodelling and dilatation after myocardial infarction (1-4). Cardiomyocyte apoptosis was found to contribute independently to the evolution of infarct size (1). Studies in the ventricular cardiomyocytes of the hearts of patients who died of acute myocardial infarction have shown that apoptosis plays a role in the process of cell death of cardiomyocytes. Saraste *et al* . (2) showed, in myocardial samples obtained from patients who died of acute myocardial infarction, that in addition to overt necrosis, a subset of cardiomyocytes undergoes apoptosis during ischemia/reperfusion injury. The apoptotic cardiomyocytes were most prominent in the border zones of recent infarction, whereas very few apoptotic cells were present in the remote non-infarcted myocardium. The time course of cardiomyocyte apoptosis after myocardial infarction remains unclear. In a previous study, Kajstura and colleagues (1) reported that dUTP-stained cardiomyocyte nuclei were not observed in sham-operated control animals and in infarcted rats 20 minutes and 1 hour. Labelled cardiomyocytes were detected first at 2 hours and did not vary at 3 hours, increased markedly at 4.5 hours, and decreased gradually at 6 hours and sharply at 7 days after coronary artery occlusion. Zhu *et al*.(3), however, reported that the strongest staining of apoptosis was detected in rats 3 days post operation. Weak staining was found 1-day and 7-day post myocardial infarction. Very few apoptotic cells were detected in the rats 2 weeks after myocardial infarction. In the present study, we have shown that cardiomyocyte apoptosis increases significantly at 24 hours after rat myocardial infarction. In all animals, clusters of apoptotic cardiomyocytes were detected particularly in the border zones of infarcted myocardium. Many apoptotic cells were also present in the endocardial regions adjacent to infarction. Therefore, it was suggested that apoptosis is the major determinant of infarct size. The reason of enhancing apoptosis in the border zones of infarcted myocardium may be hypoxia and ischemia of the myocardium. Experimental evidence suggests that cardiomyocytes are able to undergo apoptosis during hypoxia and ischemia. Factors known to associated with both ischemia-reperfusion injury and apoptosis include oxygen-derived free radicals and alterations of intracellular calcium homeostasis (18, 19). In the present study we also show that pretreatment with CT-1 for 7 days is capable of significantly reducing cardiomyocyte apoptosis, suggesting that CT-1 has a protective effect for the heart. The cardioprotection of CT-1 may be realized by means of improving hemodynamics, stimulating cardiomyocyte hypertrophy, inhibiting P53, Fas and Bax expression, and increasing Bcl-2 expression. In a previous study, Sheng *et al*. (12) have demonstrated that CT-1 promotes cardiomyocyte survival via the activation of an antiapoptotic signaling pathway that requires mitogen-activated protein kinases, whereas the hypertrophy induced by CT-1 may be mediated by alternative pathways, e.g. Janus kinase/signal transducer and activator of transcription or MEK kinase/c-Jun NH 2-terminal protein kinase (11, 20).

Apoptosis is a morphologically distinct, genetically controlled type of cell death. Extensive fragmentation of genomic DNA into nucleosome-sized fragments

by endogenous endonucleases is the biochemical hallmark of apoptosis. Apoptosis can be influenced by a wide variety of regulatory stimuli. Apoptotic genes may have an important function in regulating apoptosis. One hypothesised general molecular mediator of hypoxia induced apoptosis is the tumor suppressor transcription factor P53. Exposure of cardiomyocytes to hypoxia for 48 hours resulted in intranucleosomal cleavage of genomic DNA characteristic of apoptosis and was accompanied by increased P53 transactivating activity and protein accumulation (21).Upregulation of P53 can induce cardiomyocyte apoptosis by stimulating the expression of Bax and/or repression of Bcl-2 expression. In contrast, in an *in vivo* model of apoptosis in wild-type mice and homozygous P53 knockout mice, it was found that ischemia induced apoptosis occurred as readily in the P53 knockout mice as in wild-type mice (22). This finding indicates the existence in the heart of other, P53 independent, mechanisms of postischemic apoptosis. Results in this investigation show that the expression of P53 in cardiomyocytes of the left ventricular free wall increased significantly at 24 hours after coronary artery occlusion, and this phenomenon indicates that P53 may involve in cardiomyocyte apoptosis after myocardial infarction.

Fas antigen belongs to the family of cell surface protein that includes tumor necrosis factor receptor, nerve growth factor receptor, B-cell antigen CD40, and T-cell antigen OX40 (23). Apoptosis can be induced by Fas antigen-antibody interaction in various cell lines. Tanaka *et al.*(24) showed that messenger RNA for Fas antigen was expressed in both cardiomyocytes and nonmyocytes as revealed by Northern blotting and in situ hybridization. In hypoxic conditions, Fas messenger RNA levels in cardiomyocytes were upregulated by twofold over controls, whereas those of nonmyocytes were downregulated, indicating that cardiomyocyte death by hypoxia can occur via apoptosis and that Fas antigen may be associated with the mechanism of this apoptotic process. Zhu *et al.*(3) reported that Fas gene expression was detected in the sham-operated rats and started to over-express at 12 hours (1.5-fold in the left ventricle, 2.2-fold in the septum and 2.4-fold in the right ventricle) after myocardial infarction compared to sham-operated rats. A significant overexpression of Fas gene was observed with 1.9-fold in the left ventricle and 4.7-fold in the septum compared to sham-operated rats at 72 hours. The results of the present study show that the upregulation of Fas protein was observed at 24 hours after myocardial infarction. The extent of Fas protein expression was coincident with the number of cardiomyocyte apoptosis, indicating a role for Fas protein in the regulation of ischemia induced apoptosis in rats.

Bax is a member of the Bcl-2 family and, when overexpressed, accelerates cell apoptosis by competing with Bcl-2 (25, 26). In a recent study in an animal model of myocardial infarction, Zhu *et al.* (3) reported that no Bax gene expression was found in the sham operated rats and rats 12-hour post myocardial infarction. An overexpressed Bax gene was detected at 24 hours, but its level declined at 72 hours after myocardial infarction. Gene expression level of Bax was 4.3-fold (left ventricle), 1.7-fold (septum and right ventricle) at 24 hours compared to that at 72 hours after myocardial infarction. As mentioned earlier, apoptosis is a highly regulated process in which several regulatory proteins play a part, and in which the balance between an array of regulatory proteins decides the fate of the cell. Recent studies have documented that the mechanism of apoptosis regulation of Bcl-2 and Bax not only depends on themselves' expression levels, but also the ratio of Bcl-2 to Bax. Apoptosis was inhibited when the ratio augmented, on the contrary, increased (27). While Bax-Bax homodimers act as apoptosis inducers, Bcl-2-Bax heterodimer formation evokes a survival signal for the cells. Both Bcl-2 and Bax are transcriptional targets for the tumor suppressor protein, P53, which induces cell cycle arrest or apoptosis in response to DNA damage. In all, the coordinate performance of these molecules is crucial for controlling life and death of a cell (28, 29). The current investigation shows that the expression of Bax protein was significantly increased at 24 hours after myocardial infarction. Apoptosis was accompanied by an increase in the expression of Bax, indicating a role for this protein in the regulation of ischemia induced apoptosis in rats. Pretreatment with CT-1 for 7 days is capable of significantly inhibiting Bax expression as well as reducing cardiomyocyte apoptosis.

Bcl-2 is the most important gene that inhibits apoptosis. It can inhibit cardiomyocyte apoptosis caused by both oxygen free radicals and P53. Recent report has indicated that ischemic preconditioning resulted upregulation of Bcl-2 gene. The upregulation of Bcl-2 significantly inhibited the extent of cardiomyocyte apoptosis induced by both ischemia/reperfusion (30), suggesting that Bcl-2 can protect cardiomyocytes. The present study shows that the expression of Bcl-2 protein was increased at 24 hours after myocardial infarction. Pretreatment with CT-1 for 7 days is capable of significantly increasing Bcl-2 protein expression as well as reducing cardiomyocyte apoptosis, suggesting that Bcl-2 protein has an important role in the regulation of ischemia induced cardiomyocyte apoptosis. Induction

of Bcl-2 from cardiomyocytes may become a new pathway of myocardial preservation.

In conclusion, the present study shows that pretreatment with CT-1 for 7 days is capable of significantly improving hemodynamics and cardiac function ; reducing cardiomyocyte apoptosis ; inhibiting myocardial P53, Fas and Bax expression ; and increasing Bcl-2 expression in the rat model of acute myocardial infarction. CT-1 has a possible cardioprotective effect in this model. At present, however, the exact mechanism by which CT-1 causes this cardioprotective effect is still needed to further determine.

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