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ATTENUATION OF ISCHEMIC MYOCARDIAL INJURY AND DYSFUNCTION BY CARDIAC FIBROBLAST-DERIVED FACTOR(S)

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Abstract : Fibroblasts, the majority of non-cardiomyocytes in the heart, are known to release several kinds of substances such as cytokines and hormones that affect cell and tissue functions. We hypothesized that undefined substance(s) derived from cardiac fibroblasts may have the potential to protect against ischemic myocardium. To assess our hypothesis, using rats, we investigated : 1) the effect of cardiac fibroblast-conditioned medium (CM) on the viability of hypoxic cardiomyocytes *in vitro*, 2) the effect of CM on left ventricular (LV) function in global ischemia-reperfusion in an *ex vivo* model, 3) the mechanism underlying cardioprotection by CM. Seventy-two hours after starting a hypoxic culture, the viability of cardiomyocytes was higher ($P<0.05$) in the CM treated group (41.4%) compared to the control (20.5%). In Langendorff's preparation, 30 min after ischemia-reperfusion, LV end-diastolic pressure was lower, and LV developed pressure and $-LVdP/dt$ were higher ($P<0.01$ or $P<0.05$) in the CM group than in the control, although coronary flow did not differ between the two groups. Pretreatment with a protein kinase C inhibitor or a mitochondrial ATP-sensitive K^+ channel blocker attenuated these changes of LV function in the CM group. Such cardioprotection was achieved by a fraction of the CM having a molecular weight (MW) $> 50,000$, but not by that of the CM with a lower MW. In addition, a specific antibody against hepatocyte growth factor (HGF, MW is 84,000) did not reduce the cardioprotection afforded by CM. There may be an unknown cardioprotective substance other than HGF in rats, which mimics ischemic preconditioning and has $MW>50,000$.

Key words : Cardiomyocytes, Fibroblast, Ischemia, Hypoxia, Rat

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INTRODUCTION

In myocardial tissue, the muscle cells (cardiomyocytes) occupy more than 90% of the tissue mass. It has been reported that non-cardiomyocytes account for about 50% of the cell number despite their low mass^{1,2)}. Most non-cardiomyocytes are fibroblasts, which are known to possess a variety of activities such as the integration of myocardial tissue by producing extracellular matrix. Fibroblasts are also known to release several kinds of substances such as angiotensin II (AT II)³⁾, cardiotrophin-1 (CT-1)⁴⁾, endothelin-1 (ET-1)⁵⁾, and leukemia-inhibitory factor (LIF)⁶⁾. The main signal pathways downstream of AT II and ET-1 are phospholipase C (PLC) /phosphatidylinositol 3-kinase (IP3) and diacylglycerol (DG) / protein kinase C (PKC)⁷⁾. The receptors for LIF and CT-1 are members of a gp130 family that activates the JAK/STAT pathway^{8,9)}. These signaling pathways are considered to promote cell survival as well as to lead to cellular hypertrophy in the short term¹⁰⁾. Therefore, it is plausible that certain cardiac fibroblast-derived signal(s) may protect cardiomyocytes in a critical situation such as ischemia that causes cellular death. However, to our best knowledge, this possibility has not yet been verified.

In the present study, we hypothesized that certain factors derived from non-cardiomyocytes of the heart, mainly fibroblasts, attenuate myocardial injury due to hypoxia or ischemia-reperfusion. To evaluate this, we used a supernatant of the cultured fibroblasts, which may contain substances from cardiac fibroblasts, adding to 1) the cultured cardiomyocytes under hypoxia and to 2) the beating hearts of Langendorff's preparations as a model for whole-heart ischemia-reperfusion injury. Then we assessed their effects on the fate of cardiomyocytes in a hypoxic condition and on cardiac function after ischemia-reperfusion, and also investigated the intracellular signaling pathways involved.

MATERIALS AND METHODS

The present study with rats conformed to the Guideline on Animal Experiments of Fukushima Medical University, the Japanese Government Animal Protection and Management Law (No. 115), and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

1. Cardiomyocyte isolation and culture conditions

Cardiomyocytes and non-cardiomyocytes were isolated from the hearts of neonatal Wistar rats as described previously¹¹⁾. Briefly, those hearts were minced with scissors and the cells were dissociated with 0.4 mg/mL type II collagenase (C-6885, Sigma, Japan) and 0.6 mg/mL pancreatin (Nakarai, Osaka, Japan) in a calcium-free isotonic salt buffer (NaCl 116 mmol/L, KCl 5.4 mmol/L, MgSO₄ 5.4 mmol/L, Na₂HPO₄ 0.9 mmol/L, HEPES 25 mmol/L, glucose 5 mmol/L, pH 7.4). The dispersed cells were plated on culture dishes for 120 min at 37°C. During this step, most non-cardiomyocytes adhered to the bottom of the dishes. Non-adherent cells were collected as cardiomyocytes, and incubated in gelatin-coated 24-well culture plates at 1×10^5 cells/mL. Cells were maintained with Dulbecco's modified eagle

medium (DMEM) supplemented with 10% fetal calf serum (FCS), 50 U/mL penicillin G, 50 $\mu\text{g/mL}$ streptomycin, and 10^{-5} mol/L cytosine arabinoside (Ara-C) to eliminate any contaminated non-cardiomyocytes¹²⁾. After a 48-hour incubation with Ara-C, the original culture media was exchanged for media without Ara-C. Sarcomeric α -actin antibody (DAKO, Japan) staining showed that the isolated cardiomyocytes were 95% pure. Hypoxic culture was performed with an anoxia bag kit (Gaspak Pouch, Becton Dickinson, NJ, USA) that catalytically reduced the oxygen concentration to less than 10 ppm in 100 min¹³⁾. The survivability of cardiomyocytes was evaluated with the Trypan blue (GIBCO, NY, USA) exclusion test. Since cardiomyocytes were isolated freshly in each experiment, survival rates of the cells after 72 hours of hypoxic culture varied slightly in each experiment (e.g. Fig. 2C ; $20.5 \pm 4.2\%$, Fig. 3A ; $16.1 \pm 1.2\%$, and Fig. 3B ; $20.2 \pm 3.0\%$ each in control).

2. Preparation of cardiac fibroblast-conditioned medium (CM)

Non-cardiomyocytes (mainly fibroblasts) were cultured for 3 days with DMEM supplemented with 10% FCS, 50 U/mL penicillin G and 50 $\mu\text{g/mL}$ streptomycin at 37°C in 95% air and 5% CO_2 . These cells were passed by treatment with 0.1% trypsin (GIBCO) and seeded into 10-cm culture dishes. After 5-7 days of culture, subconfluent cells were washed twice vigorously with PBS and incubated in the serum-free DMEM for 48 hours. Then the supernatants were collected as cardiac fibroblast-conditioned media (CM) and stocked at -20°C until needed for experiments. This method allowed the cell population to reach a fibroblast level of $>95\%$ ¹⁴⁾. In preliminary studies, we performed immunostaining with anti-rat α -sarcomeric actin antibodies to identify myocytes and with the von Willebrand factor (EPOS, DAKO, Japan) to screen endothelial cells. As a result, we detected no von Willebrand factor-positive cells or α -sarcomeric actin-positive cells in cultured dishes.

For characterization of the cardiomyocyte protective factor(s), the centrifugal filter kits (MACROSEP 50K ; PALL, Gelman Laboratory, USA) were used to separate the substances in CM at 50,000 in molecular weight (MW).

3. Effect of anti-hepatocyte growth factor (HGF) antibody

We tested the effect of anti-HGF neutralizing antibody¹⁵⁾ in hypoxic cultures of cardiomyocytes with CM or control medium. Before starting the hypoxic culture, anti-HGF antibodies (0.5 and 5.0 $\mu\text{g/mL}$) were co-incubated with control medium and CM at 37°C for 60 min. We also took measurements of HGF in CM by ELISA¹⁶⁾.

4. Langendorff's preparations

Male Wistar rats at the 11-12 weeks of age ($n=82$) were anesthetized intraperitoneally (i.p.) with sodium pentobarbital 30 $\mu\text{g/g}$, heparinized (1.5 U/g, i.p.), and the beating hearts were then excised. The aorta was quickly connected to the Langendorff's apparatus, suspended, and perfused with Krebs-Henseleit buffer (NaCl 118 mmol/L, KCl 4.7 mmol/L, MgSO_4 1.2 mmol/L, KH_2PO_4 1.2 mmol/L, CaCl_2 1.8 mmol/L, NaHCO_3 25 mmol/L, glucose 11 mmol/L, pH 7.4), oxygenized with 95% O_2 and 5% CO_2 , and warmed at a constant temperature of 37°C ¹⁷⁾. The left atrium was opened and a latex balloon was inserted into the left ven-

tricle (LV) to determine the left ventricular end-diastolic pressure (LVEDP). The electrodes of the electric pulse stimulator (SEN-2201 ; Nihon Kohden, Japan) were then attached to the atria and paced at 300 bpm. After this, saline was infused into the balloon to an intra-balloon diastolic pressure of 10 mm Hg, which we considered to be our initial LVEDP. The hearts were perfused at a constant aortic pressure of 80 mm Hg, and the coronary flow was continuously monitored by an electromagnetic blood flowmeter (Nihon Kohden, Japan). By subsequently measuring the heart weight, the coronary flow per wet gram was obtained.

The heart was kept perfused for about 15 min to obtain equilibrium. After reaching the steady-state condition, the ischemia-reperfusion studies described below were initiated.

5. Myocardial ischemia-reperfusion

The rats in Langendorff's preparations ($n=82$) were divided into the following subgroups based on pretreatments used (Fig. 1). In 17 rats of control group, the perfusate of the hearts maintained at a perfusion pressure of 80 mm Hg was replaced by the oxygenized control medium (DMEM) at 37°C and bubbled with 95% O₂ and 5% CO₂ for 10 min. For fibroblasts-conditioned medium (CM)-treated group (CM group) including 14 rats, the control medium was replaced by oxygenized CM kept at 37°C and bubbled with 95% O₂ and 5% CO₂ for 10 min. In two groups ($n=8$, each), the perfusate was replaced with the control or the CM containing chelerythrine (an inhibitor of PKC¹⁸) 6.4 μ mol/L each for 10 min. In two other groups, the perfusate was switched to either the oxygenized control medium ($n=8$) or the CM ($n=11$) containing 5-hydroxydecanoate (5-HD, a blocker of mitochondrial ATP-

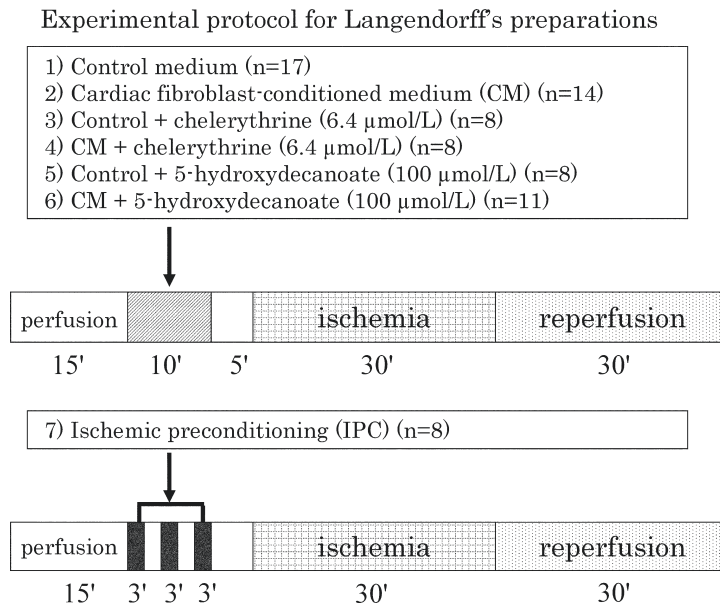


Fig. 1. Experimental protocol for Langendorff's perfusion. All hearts were perfused for 90 min, consisting of a 30-min pre-ischemic period followed by a 30-min global ischemia and 30-min reperfusion. For pre-ischemic period, all hearts were perfused with control medium (DMEM) for 15 min, followed by the treatment for each group.

sensitive K^+ channel)¹⁹ 100 μ mol/L each for 10 min. As a positive control for cardioprotection, the process of global ischemia induced by a total flow stop for 3 min followed by a reflow for 5 min was repeated 3 times to achieve ischemic preconditioning (IPC) in the control medium-perfused hearts (IPC group, $n=8$). These pre-treatments were completed 5 min before starting the global ischemia-reperfusion. After each pretreatment, the rat heart suffered global ischemia by total flow stop for 30 min. During this ischemic period, atrial pacing was discontinued. Then, 3 min after reperfusion, with the perfusate at a constant perfusion pressure of 80 mm Hg, the heart was paced again at 300 bpm. Heart rate, LV peak systolic pressure [LVPSP (mm Hg)], LVEDP (mm Hg), LV developed pressure [LVDeVP; LVPSP-LVEDP (mm Hg)], and maximal $\pm$ LVdP/dt (mm Hg/sec, each) were continuously monitored using a polygraph system (Nihon Kohden, Japan) from the beginning of the study until 30 min after each reperfusion. Following that, the hearts were perfused for an additional 1.5 hours for histopathological assessment.

The sham group ($n=8$) exempt from ischemia-reperfusion procedures was perfused with oxygenized Krebs-Henseleit buffer throughout the study.

6. Assessment of infarct size

Two hours after reperfusion, the perfusate was changed to 2% triphenyl tetrazolium chloride (TTC) solution warmed at 37°C, and was perfused at a pressure of 80 mm Hg for 5 min. It was then detached from the Langendorff's apparatus, again stained with a TTC solution in the dish at 37°C for an additional 30 min, after which the heart weight was measured and the left ventricle was cut into three 3 mm-thick short-axial slices. On the upper and lower sides of the middle LV slice, the total area of the LV wall and the infarcted area (TTC unstained) were stereoscopically assessed by the point-counting method of Weibel²⁰. The infarct size was calculated as : counts of TTC-unstained area/counts of total LV wall area (%) on short-axial middle LV myocardial slices.

7. TUNEL staining

After counting of TTC-stained and -unstained area, the slices of the heart tissue were fixed with 10% neutral-buffered formalin, the middle portions were embedded in paraffin, and double-stained with terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) to identify TUNEL-positive nuclei of cardiomyocytes, and with methylgreen to determine the total number of nuclei on myocardial sections as reported previously²¹. Light microscopic counting of TUNEL-positivity (%) [number of TUNEL-positive cardiomyocytes/number of methylgreen-positive total cardiomyocytes $\times$ 100 on short-axial myocardial sections] was performed at $\times$ 200 magnification using the instrument's eyepiece for the point-counting method, as reported previously²¹.

8. Statistical analysis

Data are presented as mean $\pm$ SEM. Statistical analysis was performed by two-way analysis of variance. If F test results were <0.05 , Bonferroni's post hoc test was performed. A P value of less than 0.05 was considered significant.

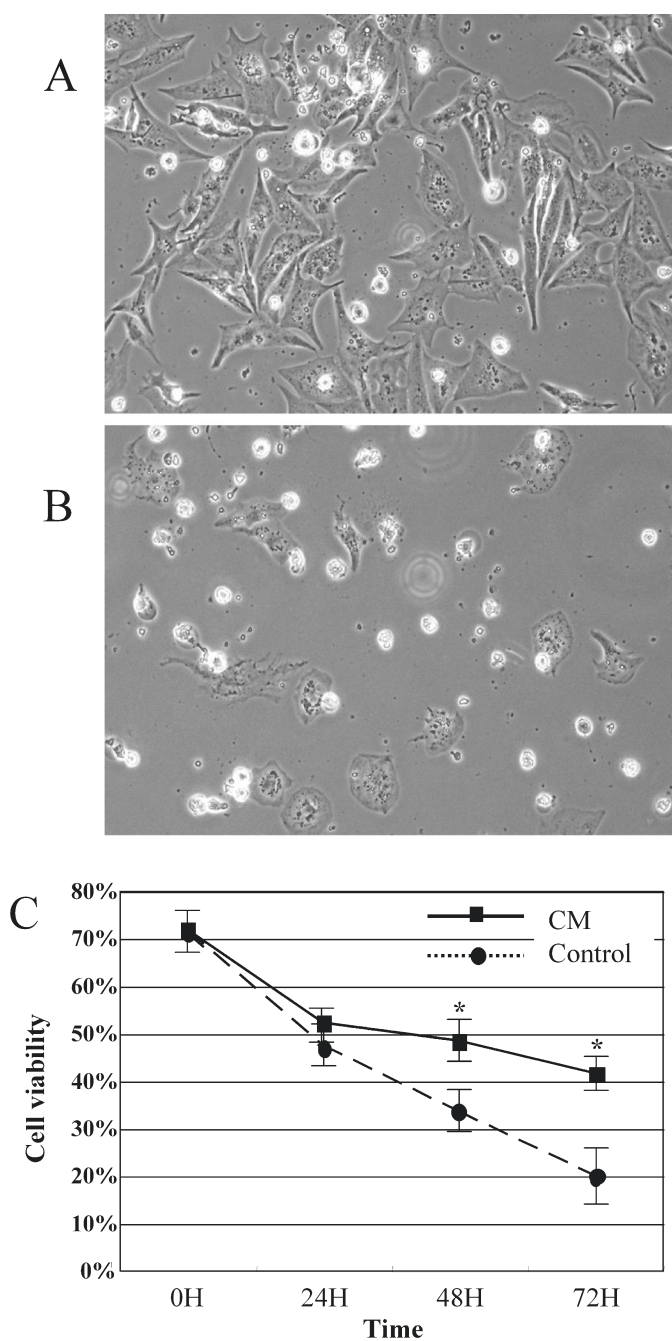


Fig. 2. Effects of cardiac fibroblast-conditioned medium (CM) on rat neonatal cardiomyocyte survival under hypoxic culture conditions. A : Phase contrast image of cardiomyocytes treated with CM for 72 hours. B : Representative morphology of cardiomyocytes in control medium after 72 hours. Fewer live cells (compared to CM group) were observed. C : Time course of cardiomyocyte viability under hypoxic culture conditions. The viability of cardiomyocytes (live or dead) was evaluated with the Trypan blue exclusion test. * $P < 0.05$ versus control medium.

RESULTS

1. *Effect of cardiac fibroblast-conditioned media (CM) on isolated cardiomyocytes in hypoxia*

Isolated cardiomyocytes were cultured with or without fibroblast-conditioned medium (CM) under hypoxic condition. The hypoxic condition reduced live cells, which kept rod-like or polygonal shapes attached to the bottom of culture dishes as shown in Fig. 2A, and increased dead cells that shrunk and became detached from the surface of the dishes (Fig. 2B), in a time dependent manner. Trypan blue staining revealed that the survival rate of CM-treated cardiomyocytes became significantly higher than that of untreated after 48 hours (Fig. 2C).

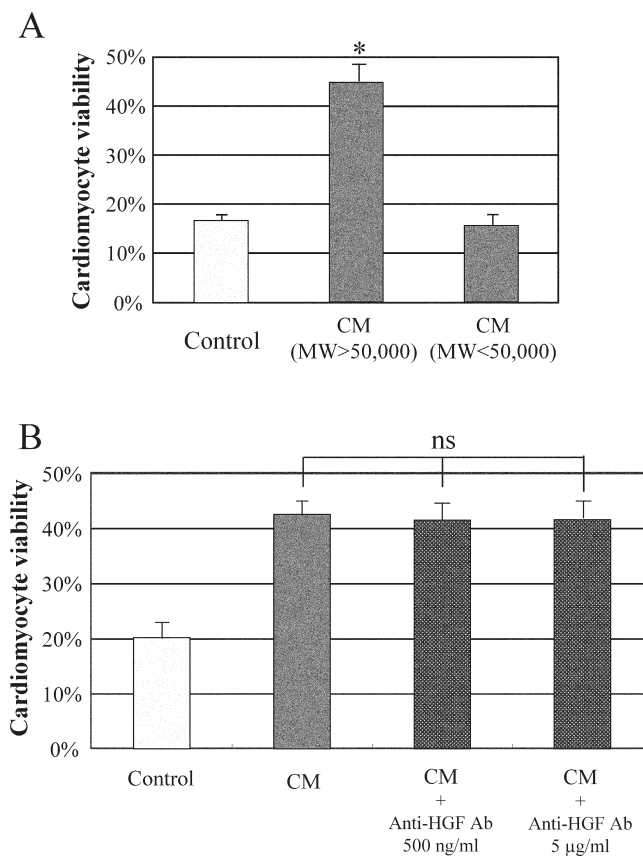


Fig. 3. A : Cardiomyocyte survival after a 72-hour hypoxic culture. The viability of cardiomyocytes was assessed with the Trypan blue staining. Cardiomyocyte protective activity occurred in fractions of molecular weight greater than 50,000. * $P < 0.05$ versus control medium. B : Cardiomyocyte viabilities after 72 hours of hypoxic culture. Amelioration of cardiomyocyte survival with CM was not diminished by two different doses of anti-HGF neutralizing antibody.

2. Molecular weight of fibroblast-derived factor and relation to HGF

The centrifugal filtering method revealed that cardiomyocyte protective activity was observed only in fractions of molecular weight (MW) above 50,000 (Fig. 3A). Because MWs

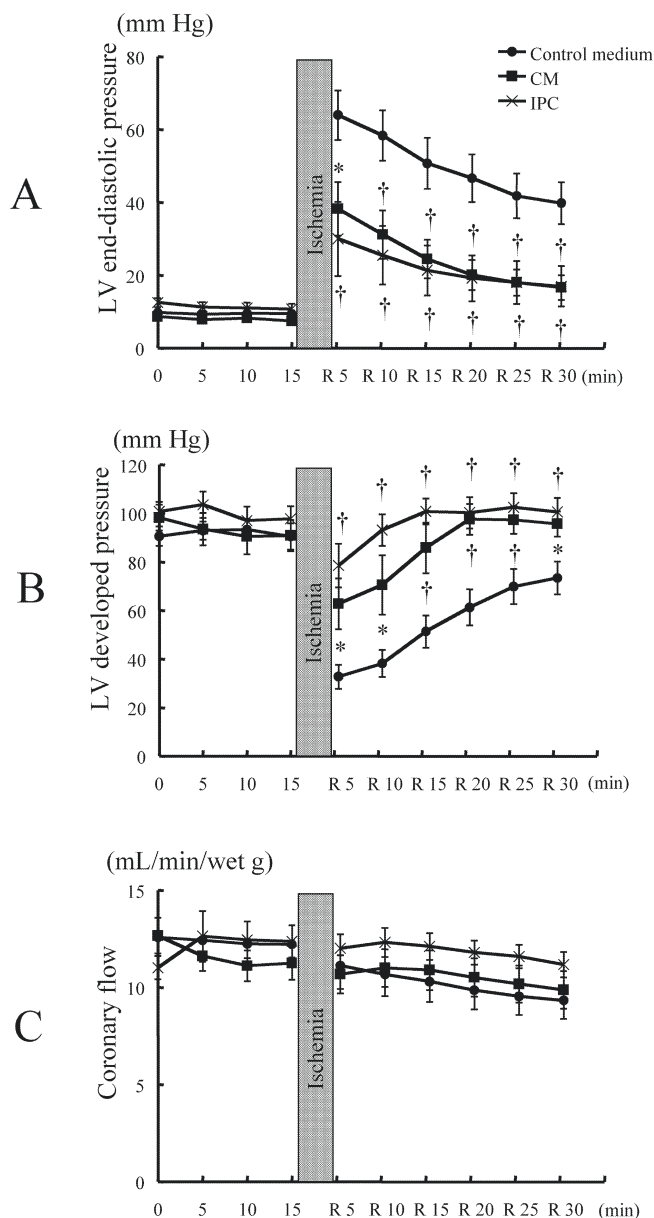


Fig. 4. Parameters at perfusion pressure of 80 mm Hg in Langendorff's preparations before and after ischemia. A: Effects of control medium, CM, or ischemic preconditioning (IPC) on LV end-diastolic pressure. B: Effects of control medium, CM, or IPC on LV developed pressure. C: Effects of control medium, CM, or IPC on coronary flow. Data are mean $\pm$ SEM. * P < 0.05, † P < 0.01 versus control medium. 'R' indicates reperfusion.

of most known cardioprotective substances except hepatocyte growth factor (HGF) are smaller than 50,000 (described in the discussion), we needed to clarify whether or not HGF was associated with our experimental results. Treatment with anti-HGF antibody to neutralize soluble HGF did not affect the cell survival effect of CM ($42.6 \pm 3.0\%$ versus $41.4 \pm 3.5\%$ [500 ng/mL] and $41.6 \pm 4.9\%$ [0.5 $\mu\text{g/mL}$], ns. [Fig. 3B]).

3. Effects of CM on cardiac function in an ex vivo model

Among the 82 hearts in the Langendorff's study, 21 (25.6%) did not restart beating after pacing was started following ischemia-reperfusion. This incidence tended to be higher in the control medium group (35.2%) than in other groups (21.4%). These hearts that persistently lost beats were excluded from further study. In the sham group (without ischemia), LVPSP, LVDeVP, LVEDP, $\pm$ LVdP/dt and coronary flow did not change significantly between the start and end of the study (Table 1A and 1B). After reperfusion following ischemia, LVEDP in the control medium group peaked at 5 min after reperfusion (Fig. 4A), resulting in a slow increase in the LVDeVP (Fig. 4B), whereas coronary flow after reperfusion recovered similar

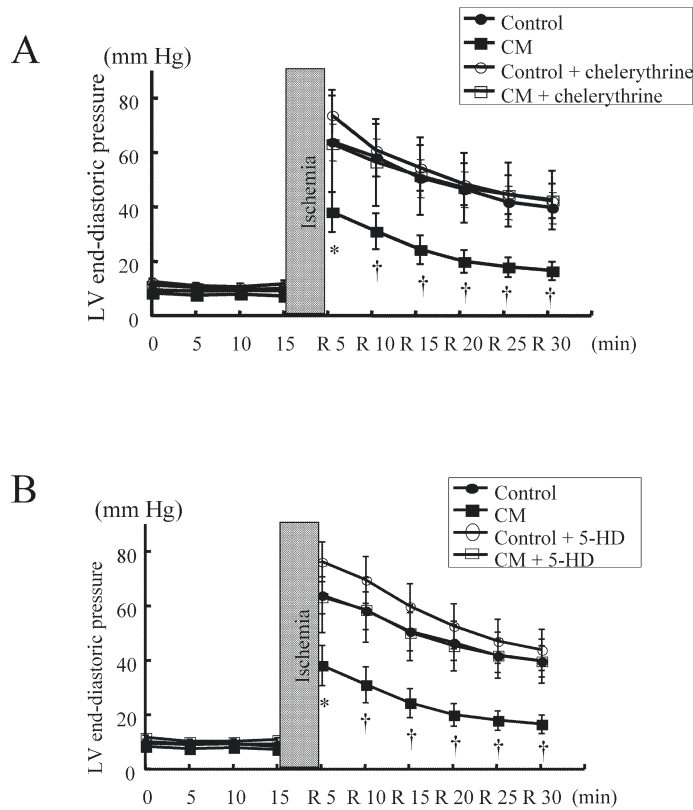


Fig. 5. LV end-diastolic pressure at perfusion pressure of 80 mm Hg in Langendorff's preparations. A : Effects of chelerythrine (PKC inhibitor) on control medium and CM. B : Effects of 5-HD (ATP-sensitive K^+ channel blocker) on control medium and CM. * $P < 0.05$, † $P < 0.01$ versus control medium. 'R' indicates reperfusion.

Table 1. Effects of various interventions on hemodynamic parameters in hearts subjected to ischemia-reperfusion in Langendorff's preparations
A : Just before ischemia

	sham without ischemia (n=8)	control medium (C) (n=17)	conditioned medium (M) (n=14)	C +chelerythrine (n=8)	M +chelerythrine (n=8)	C +5-HD (n=8)	M +5-HD (n=11)	C + ischemic preconditioning (n=8)
LVPSP (mmHg)	109 ± 4	100 ± 5	98 ± 6	119 ± 6	107 ± 9	118 ± 7	106 ± 7	108 ± 6
LVEDP (mm Hg)	10 ± 0.1	9 ± 1	7 ± 1	12 ± 1	10 ± 1	8 ± 1	11 ± 1	11 ± 1
LVD _e VP (mm Hg)	99 ± 4	91 ± 5	91 ± 6	107 ± 5	97 ± 9	109 ± 8	95 ± 7	98 ± 5
+ LVdP/dt (mm Hg/sec)	3,150 ± 216	3,084 ± 286	3,114 ± 290	3,381 ± 226	2,746 ± 573	3,376 ± 465	2,866 ± 241	3,051 ± 144
– LVdP/dt (mm Hg/sec)	2,200 ± 227	1,932 ± 93	1,964 ± 146	2,329 ± 226	2,043 ± 307	2,325 ± 119	2,114 ± 150	1,706 ± 98
CF (mL/min/wet g)	11 ± 1	12 ± 1	11 ± 1	14 ± 0.4	12 ± 1	12 ± 1	10 ± 1	12 ± 1

B : 30 minutes after reperfusion

	sham without ischemia (n=8)	control medium (C) (n=11)	conditioned medium (M) (n=11)	C +chelerythrine (n=6)	M +chelerythrine (n=6)	C +5-HD (n=6)	M +5-HD (n=7)	C + ischemic preconditioning (n=6)
LVPSP (mm Hg)	110 ± 4	113 ± 9	112 ± 6	119 ± 8	117 ± 11	124 ± 5	127 ± 8	118 ± 8
LVEDP (mm Hg)	9 ± 0.3	40 ± 6 [†]	17 ± 3§	42 ± 6 [†]	43 ± 11 [†] #	44 ± 8 [†]	40 ± 8 [†] #	17 ± 6 [‡]
LVD _e VP (mm Hg)	100 ± 4	74 ± 7 [†]	96 ± 5§	76 ± 12*	74 ± 4 [†]	80 ± 9*	87 ± 6	101 ± 6 §
+ LVdP/dt (mm Hg/sec)	3,125 ± 154	2,500 ± 408	3,036 ± 295	2,005 ± 400*	2,288 ± 196*	2,505 ± 444	2,487 ± 199	3,628 ± 216 [‡]
– LVdP/dt (mm Hg/sec)	2,288 ± 230	1,505 ± 143 [†]	1,905 ± 148 [‡]	1,517 ± 269 [†]	1,533 ± 190 [†]	1,675 ± 194*	1,779 ± 107*	2,133 ± 85 [‡]
CF (mL/min/wet g)	9 ± 1	9 ± 1	10 ± 1	9 ± 1	9 ± 1	9 ± 1	8 ± 1	11 ± 1

LVPSP ; left ventricular peak systolic pressure, LVEDP ; left ventricular end-diastolic pressure, LVD_eVP ; left ventricular developed pressure, LVdP/dt ; the first derivatives of left ventricular pressure, CF ; coronary flow, 5-HD ; 5-hydroxydecanoate.
Data are mean ± SEM. **P*<0.05, [†]*P*<0.01 versus sham, [‡]*P*<0.05, § *P*<0.01 versus control medium (C). # *P*<0.05 versus conditioned medium (M)

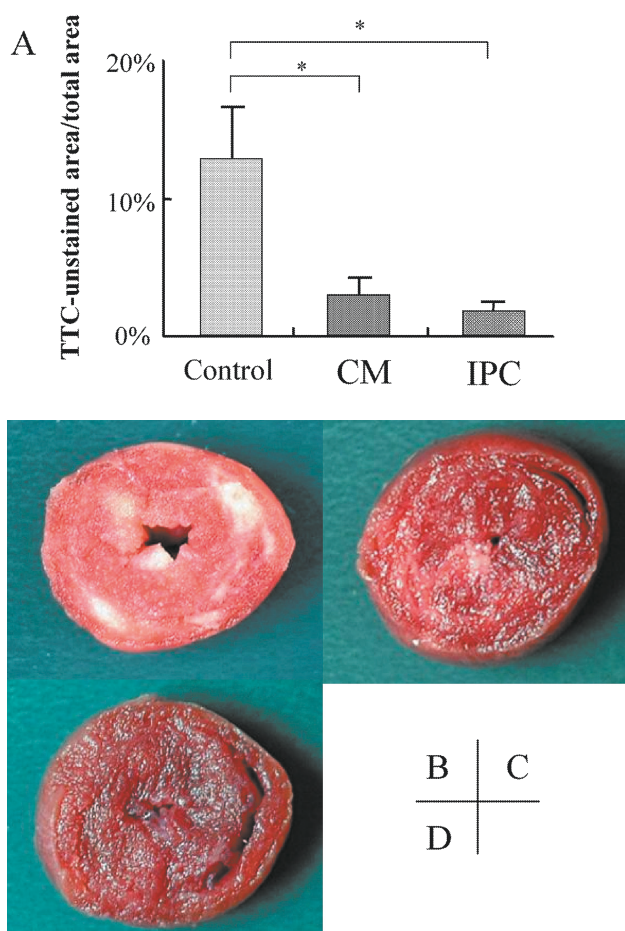


Fig. 6. A : Infarct size assessed with triphenyl tetrazolium chloride (TTC) staining in Langendorff's preparations. B-D : Representative examples of the transverse sections of rat hearts after TTC staining (B : control, C : CM, D : IPC). Whitish colors (TTC unstained) represent infarct areas. Infarct size was significantly reduced in the CM treated group and the IPC group. * $P < 0.05$ versus control.

to those of both CM and ischemic preconditioning, as well as compared to that before starting ischemia (Fig. 4C, ns except $P < 0.05$ for 25 to 30 min after reperfusion versus just before ischemia ; significant marks not shown). Such an increase in LVEDP and a decrease in LVDeVP were attenuated by the treatment with CM or ischemic preconditioning, leading to a decline in LVDeVP in these two groups.

In this model, we also analyzed the molecular weight of the factor(s) in CM showing cardioprotective activity, was smaller or greater than 50,000. Improvement of parameters was observed in the group treated with the fraction of molecular weight over 50,000 (e.g., % LVEDP elevation 10, 20, and 30 min after coronary reperfusion : whole CM ; 100%, MW < 50,000 ; $293 \pm 48.9\%$ *, MW > 50,000 ; $56.8 \pm 18.6\%$ 10 min after reperfusion, whole

CM ; 100%, MW<50,000 ; $391.3 \pm 114.0\%^*$, MW>50,000 ; $36.4 \pm 26.5\%$ 20 min after reperfusion, whole CM ; 100%, MW<50,000 ; $265.0 \pm 80.5\%^*$, MW>50,000 ; $70.0 \pm 35.0\%$ 30 min after reperfusion, $*P<0.05$ versus whole CM).

4. Influence of chelerythrine and 5-HD on the effect of CM in *ex vivo* model

To examine involvement in myocardial protection of PKC and ATP-sensitive K^+ channel, we added each inhibitor to perfusate of control group and CM group. Co-treatment with chelerythrine or 5-HD did not modify the hemodynamic parameters both in the control and CM group before myocardial ischemia (Table 1A). In contrast, the addition of chelerythrine to CM abolished the favorable effects of CM on LVEDP (Fig. 5A), LVDeVP and $-LVdP/dt$ resulting in the values similar to those of control group (Table 1B). 5-HD also reversed the favorable changes in LVEDP (Fig. 5B), LVDeVP and $-LVdP/dt$ induced by CM (Table 1B).

5. Infarct size and TUNEL-positivity

A TTC-unstained area was not found in the sham rats. As shown in Fig. 6, CM reduced infarct size from $12.6 \pm 4.0\%$ (control) to $2.8 \pm 1.4\%$ (CM) at the end of the Langendorff's study. Similarly, ischemic preconditioning achieved an infarct size reduction ($1.4 \pm 0.3\%$). TUNEL-positivity was $0 \pm 0\%$ in the sham group, $7.0 \pm 1.9\%$ ($P<0.01$ versus sham group) in the control group, $1.2 \pm 0.6\%$ ($P<0.05$ versus control medium group) in the CM group, and $0.4 \pm 0.1\%$ ($P<0.01$ versus control medium group) in the ischemic preconditioning group (data not shown).

DISCUSSION

Interactions occur between cardiomyocytes and fibroblasts in the heart^{4,22}. In this study, we demonstrated that the cardiac fibroblast-conditioned medium (CM) ameliorates the survival of cardiomyocytes in hypoxia. CM consists of substances from cultured cardiac fibroblasts. We further showed that CM attenuates LV dysfunction after ischemia-reperfusion in the *ex-vivo* model. PKC activation and mitochondrial ATP-sensitive K^+ channel opening seem to be involved in the mechanisms of this heart-protective action.

1. Cardiomyocyte and heart protection by the CM

We found that the supernatant of cultured cardiac fibroblast had the potential to rescue cardiomyocytes from hypoxic damage (Fig. 2). This effect was observed with CM that was prepared by a 12-hour or longer incubation (data not shown), suggesting that cardiac fibroblasts produced some cardioprotective substance and released it into the supernatant during the incubation. This cardioprotective action via cardiac fibroblast-releasing substance(s) was also observed in the whole heart. In Langendorff's preparations, ischemia-reperfusion using the control medium caused increases in LVEDP and decreases in LVDeVP and $-LVdP/dt$, whereas pre-treatment of CM ameliorated these functional parameters. From these results, CM improved predominantly diastolic dysfunction in this *ex-vivo* model. Since we have not analyzed the effect of CM in other models, it remains to be determined whether CM has the

potential to improve systolic dysfunction in heart failure.

As shown in Fig. 4B, LVDeVP after reperfusion was lower in the control group than in the other two groups. In contrast, coronary flow was similar among the 3 groups (Fig. 4C). From the present data, lower LVDeVP in the control medium group was derived from higher LVEDP, and LVPSP was similar among the three groups. Accordingly, myocardial O₂ demand after reperfusion may not largely differ among these groups, probably resulting in similar coronary flow. Thus, in these three groups, the recovery of coronary flow after reperfusion close to the pre-ischemic level seems to reflect no significant decrease in LVPSP.

2. PKC and mitochondrial ATP-sensitive K⁺ channel pathways

PKC activation and subsequent opening of mitochondrial ATP-sensitive K⁺ channel have been reported to promote cell survival in the ischemia-reperfusion condition as shown in the mechanisms of ischemic preconditioning²³. We assessed whether PKC-mitochondrial ATP-sensitive K⁺ channel pathways are involved in the mechanisms of cardioprotection by CM, since the ischemic preconditioning is one of the most potent way for protecting cardiomyocytes from ischemia, and that several plausible candidates that cardiac fibroblast can release, may also share this pathway (e.g., AT II²⁴, ET-1²⁵, LIF²⁶ and CT-1²⁷). In fact, cardioprotection against ischemia-reperfusion by CM seemed to be associated with PKC activation, and this cardioprotective effect, especially on LVEDP, was reversed by chelerythrine, a PKC inhibitor (Fig. 5A). Furthermore, a blockade of the effect of CM by 5-HD suggested the involvement of mitochondrial ATP-sensitive K⁺ channel opening in the mechanisms of this cardioprotection (Fig. 5B).

3. Plausible substance(s) involved in myocardial protection

An anti-apoptotic pathway is the common cascade involved in the cell survival signals by different factors²⁸. Although TUNEL-positivity does not necessarily equate with apoptosis, it is plausible that apoptosis is involved in the infarct formation²⁹. Our study revealed that the cardioprotective effect of cardiac fibroblast-derived substance(s) might partially mediate an anti-apoptotic cascade (Fig. 6). If the cardioprotection afforded by CM is attributed to the effects produced by known diffusible substance(s) that can be released from cardiac fibroblasts, the candidates responsible may be cardioprotective peptides such as AT II and ET-1, and cytokines including LIF and CT-1, as mentioned above. In addition, various other substances, including adenosine³⁰, opioids³¹, and nitric oxide³² have been reported to possess the capability of cardioprotection by IPC. These substances also need to be taken into consideration as possible candidates for cardioprotection. However, the centrifugal filtering method showed that MW of the relevant factor was greater than 50,000. The smaller fraction in CM had no effect either on the cardiomyocyte-survival or the whole heart functions in *ex-vivo* model. These results exclude lower MW substances, including nitric oxide (gas), adenosine (0.27 K), AT II (1.05 K), ET-1 (2.49 K), opioids (e.g. rat β -endorphin : 3.47 K), LIF (19.7 K), and CT-1 (21.5 K) as candidates for cardioprotective action.

Hepatocyte growth factor (HGF) was originally identified as a potent mitogen of mature hepatocytes³³, with a molecular weight of 84 kD in a mature form. HGF has been reported

to be having potential for cardioprotection^{15,34}). Although HGF is greater than 50,000, the concentration of HGF in the CM was under measurement sensibility [measured by ELISA in Osaka University¹⁶]. In addition, as shown in Fig. 3B, the cardiomyocyte-protective activity of the CM was not canceled by enough amount of anti-HGF neutralizing antibody. From these results, HGF is not responsible for cardioprotection at least in our models. Further studies to identify ligands in CM would shed more light on the molecular network in the self-defense systems in cases of heart ischemia

4. *Clinical relevance*

Our results have clinical implications. First, the present study sheds light on another role of cardiac fibroblasts as a protector of myocytes in ischemic myocardium. The increases in cardiac fibroblasts occur in many pathologic conditions of the myocardium including ischemia. Therefore, it is of interest that such cardioprotection by non-cardiomyocytes functions also in the abnormal condition with proliferation of fibroblasts. Second, the substance(s) contained in the cardiac fibroblast-derived CM may be as yet unknown factor(s) distinct from the known bioactive peptides or cytokines mentioned above. Thus, their determination may lead to the development of novel strategies for cardioprotection.

5. *Study limitations*

Our study has limitations. First, we did not determine the mechanisms of cardioprotection in detail, especially regarding the upstream cascades of PKC activation as well as pathways other than PKC. In addition, the cardioprotective substance(s) contained in CM have not been identified as mentioned above. Second, it is of interest to note the effects of the combined treatment by ischemic preconditioning and cardiac perfusion with CM in Langendorff's preparation, which may provide a clue to experiments designed to investigate CM's cardioprotective mechanisms. However, we have not undertaken such a series of experiments.

CONCLUSIONS

In this study, cardiomyocyte survival in hypoxia was improved by CM. Furthermore, CM attenuated whole heart dysfunction due to ischemia-reperfusion in Langendorff's model. This cardioprotective effect was at least partly mediated by PKC activation and a mitochondrial ATP-sensitive K⁺ channel opening. These results suggest a role for non-cardiomyocytes, especially fibroblasts, in mitigating injury to cardiomyocytes in hypoxia and myocardial ischemia-reperfusion.

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