

PRE-CLINICAL RESEARCH

Ischemic Pre-Conditioning Enhances the Mobilization and Recruitment of Bone Marrow Stem Cells to Protect Against Ischemia/Reperfusion Injury in the Late Phase

Takahiro Kamota, MD, Tao-Sheng Li, MD, PHD, Noriyasu Morikage, MD, PHD, Masanori Murakami, MD, PHD, Mako Ohshima, MS, Masayuki Kubo, PHD, Toshiro Kobayashi, MD, PHD, Akihito Mikamo, MD, PHD, Yasuhiro Ikeda, MD, PHD, Masunori Matsuzaki, MD, PHD, Kimikazu Hamano, MD, PHD

Ube, Yamaguchi, Japan

Objectives	The aim of this study was to investigate whether the mobilization and recruitment of bone marrow stem cells (BMSCs) contribute to cardioprotection in the late phase after ischemic pre-conditioning (IPC).
Background	IPC is an innate phenomenon in which brief exposure to sublethal ischemia provides tissue protection from subsequent ischemia/reperfusion (I/R) injury. A delayed cardioprotection also occurs after IPC, but the precise mechanism is unclear.
Methods	IPC was created with 4 cycles of 5-min occlusion and reperfusion of the abdominal aorta in mice. Heart I/R injury was induced by occluding the left anterior descending artery for 30 min immediately (early phase) or 24 h (late phase) after IPC.
Results	Serum vascular endothelial growth factor and stromal cell-derived factor-1 α levels were increased significantly 1 and 3 h after IPC, but CD34+ and CD34+/flk-1+ stem cells in the peripheral blood were increased significantly 12 and 24 h after IPC ($p < 0.05$). Compared with the control treatment, both the early and late phases of IPC protected the heart against I/R injury. However, the recruitment of BMSCs was significantly greater in the heart when I/R injury was induced in late phase than in the early phase of IPC ($p < 0.01$). Interestingly, the blockade of the recruitment of BMSCs significantly attenuated the cardioprotective effect of IPC in the late phase ($p < 0.01$) but did not change in the early phase.
Conclusions	Cardioprotection was observed in the early and late phases of IPC; however, the enhanced mobilization and recruitment of BMSCs played an important role in the late phase of IPC. (J Am Coll Cardiol 2009;53:1814-22) © 2009 by the American College of Cardiology Foundation

Ischemic pre-conditioning (IPC) is a well-known innate phenomenon in which brief exposure to sublethal ischemia provides tissue protection from subsequent ischemia/reperfusion (I/R) injury in various organs (1). IPC has also been found to exert systemic effects that result in protection from I/R injury of tissues remote from those undergoing pre-conditioning. This is termed "remote IPC" (2). The protective effect after IPC occurs in 2 distinct phases (3): the

early phase develops rapidly from the time of the initial ischemic insult and lasts for 2 to 3 h, and the late phase develops 12 to 24 h after the initial insult and persists for 3 to 4 days (4).

The mechanisms of the 2 phases of IPC are completely different. The early phase of IPC is generally thought to be dependent on the immediate release of mediators such as adenosine and bradykinin, the rapid post-translational modification of pre-existing proteins, and the activation of complex second messenger systems (5). Conversely, the late phase of IPC is thought to be related to the transcriptional activation of various genes including nuclear factor-kappa B and signal transducer and activator of transcription-3 that in turn cause the synthesis of new cardioprotective proteins (5-8). This explains the delayed and prolonged time course

From the Department of Surgery and Clinical Science, Department of Medicine and Clinical Science, Yamaguchi University Graduate School of Medicine, Ube, Yamaguchi, Japan. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture. Drs. Kamota and Li contributed equally to this work.

Manuscript received September 30, 2008; revised manuscript received February 10, 2009, accepted February 16, 2009.

of protection in the late phase of IPC; however, the precise mechanism of organ protection by IPC remains unclear, especially in the late phase.

Although the delayed protection of IPC was generally thought to be mediated by the activation of various transcription factors and the synthesis of new proteins, expression of some cardioprotective factors has not revealed a second increase peak during the late phase of IPC (9,10). Interestingly, recent investigations have found the mobilization of bone marrow stem cells (BMSCs) into circulating blood, hours to days after IPC (11,12). Furthermore, many growth factors released by BMSCs have been found to protect cardiomyocytes against apoptosis (13), and BMSCs are now used effectively for myocardial repair (14,15). Considering the time needed to induce the mobilization of BMSCs into circulating blood, we hypothesized that the mobilized circulating BMSCs would be recruited into the damaged heart and contribute to the delayed protection of IPC.

In this study, we monitored the changes in cardioprotective factors and the mobilization of BMSCs after remote IPC. Then, with a heart I/R injury model, we investigated whether the mobilized BMSCs were recruited into the heart and whether they contributed to cardioprotection, especially in the late phase of IPC.

Methods

Animals. We obtained male C57BL/6 mice and Wistar rats from Japan SLC (Shizuoka, Japan). Green fluorescent protein (GFP)-transgenic mice were kindly provided by Masaru Okabe (16). All experiments were approved by the Institutional Animal Care and Use Committee of our university.

Bone marrow transplantation (BMT). BMT was performed as described previously (17). Briefly, after lethal irradiation (10 Gy), each C57BL/6 mouse received intravenous injection with 5×10^6 bone marrow mononuclear cells collected from GFP-transgenic mice. These chimera mice were studied 8 weeks after BMT, and the GFP-positive (GFP+) circulating leukocytes were checked by flow cytometry ($93.6 \pm 2.1\%$).

Remote IPC and heart I/R injury models. A remote IPC model was established in mice as described previously (18). Briefly, we performed a laparotomy and exposed the abdominal aorta above the bifurcation. Remote IPC was induced by 4 cycles of 5-min occlusion followed by 5-min reperfusion of the abdominal aorta. Control mice received sham laparotomy with exposure of the abdominal aorta.

Heart I/R injury was induced in the BMT chimera mice immediately after IPC (early IPC group), 24 h after IPC (late IPC group), or immediately after sham laparotomy (control group). Briefly, after general anesthesia and tracheal intubation, the mice were artificially ventilated with room air. We performed a left thoracotomy and occluded the left anterior descending artery for 30 min with an 8-0 polypropylene suture (19), followed by reperfusion.

Monitoring the changes in plasma cytokines and circulating BMSCs after IPC.

After 1, 3, 6, 12, 24, 48, and 72 h of IPC, the mice were killed, and blood samples were collected. We measured the plasma concentrations of stromal cell-derived factor (SDF)-1 α and vascular endothelial growth factor (VEGF) with mouse SDF-1 α and VEGF enzyme-linked immunosorbent assay kits (R&D Systems, Inc., Minneapolis, Minnesota). Mononuclear cells were separated from the blood samples by gradient centrifugation. Cells were stained with rabbit antimouse flk-1 polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, California), followed by FITC-conjugated second antibody. After washing, cells were also stained with PE-conjugated rat antimouse CD34 monoclonal antibody (eBioscience Inc., San Diego, California). Respective isotype controls were used as a negative control. Quantitative flow cytometry analysis was done with a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey).

Echocardiography. Cardiac function was assessed 1 and 2 weeks after I/R injury by a single observer blinded to the treatment regimen, with echocardiography with an HDI-5000 ultrasound machine equipped with a 15-MHz probe (Philips Medical Systems, Eindhoven, the Netherlands) (19). After the induction of light general anesthesia, the hearts were imaged 2-dimensionally in long-axis views at the level of the greatest left ventricular (LV) diameter. This view was used to position the M-mode cursor perpendicular to the LV anterior and posterior walls. The left ventricular end-diastolic diameters (LVEDDs) and left ventricular end-systolic diameters (LVESDs) were measured from M-mode recordings according to the leading-edge method. The left ventricular fractional shortening (LVFS) was calculated as: $(LVEDD - LVESD)/LVEDD \times 100$.

Histological analysis. Mice from each group were killed 2 weeks after treatment. The hearts were harvested, and frozen sections were used for histological analysis (19). Hematoxylin-eosin and Azan staining were done to measure the wall thickness and the fibrosis area of the LV 2 weeks after treatment. With the image analysis software Image-Pro Plus (Media Cybernetics Inc., Bethesda, Maryland), the mean wall thickness was measured from 3 equidistant points in the LV anterior wall, and the area of fibrosis was calculated as the area stained blue divided by the total area of the LV wall. Mea-

Abbreviations and Acronyms

BMSC = bone marrow stem cell
BMT = bone marrow transplantation
GFP = green fluorescent protein
IPC = ischemic pre-conditioning
I/R = ischemia/reperfusion
LV = left ventricle/ventricular
LVEDD = left ventricular end-diastolic diameter
LVESD = left ventricular end-systolic diameter
LVFS = left ventricular fractional shortening
SDF = stromal cell-derived factor
TUNEL = terminal deoxynucleotidyl transferase dUTP nick end labeling
VE = vascular endothelial
VEGF = vascular endothelial growth factor

surements were done in at least 3 separated sections of each heart, and the averages were used for statistical analysis.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. To study the apoptosis of cardiomyocytes, 5 mice from each group were killed 1 day after I/R injury. The hearts were harvested, and frozen sections were used for analysis. The apoptotic cardiomyocytes were detected by TUNEL assay with an ApopTag-Red Apoptosis in situ Detection Kit (CHEMICON International Inc., Billerica, Massachusetts). Sections were also stained with 4'-6-diamidino-2-phenylindole to visualize the nuclei. At least 20 different fields were selected randomly for counting from 3 independent slides of each heart, and the averages were used for statistical analysis.

Estimation of the recruitment of BMSCs. Histological detection of the recruitment of BMSCs in the heart was done 1 day after treatment. Frozen sections were incubated with Alexa Fluor 488-conjugated rabbit anti-GFP polyclonal antibody (Invitrogen Inc., Carlsbad, California) to detect the bone marrow-derived GFP+ cells, and stem cells were then identified by staining with PE-conjugated rat monoclonal antibodies against mouse Sca-1 or c-kit. The

BMSCs were counted in at least 10 different fields from 3 independent slides of each heart, and the averages were used for statistical analysis.

The recruitment of BMSCs was also estimated by quantitative flow cytometry, as described previously (20). Briefly, we extracted the hearts 1 day after I/R injury in each group (n = 3). The myocardium from each heart was minced and then digested in 0.1% collagenase. We collected all of the isolated cells from each heart and incubated with PE-conjugated rat monoclonal antibodies against mouse Sca-1 or c-kit. Quantitative flow cytometry analysis was done as described in the preceding text.

Differentiation of BMSCs. The differentiation of BMSCs in the heart was detected 2 weeks after I/R injury. The BMSCs were stained with Alexa Fluor 488-conjugated rabbit anti-GFP polyclonal antibody (Invitrogen Inc.). The myocardial differentiation of BMSCs was identified by immunostaining with goat antitroponin I polyclonal antibody, followed by Texas Red-conjugated second antibody, and endothelial differentiation of BMSCs was identified by PE-conjugated goat antivascular endothelial (VE)-cadherin polyclonal antibody (Santa Cruz Biotechnology Inc.).

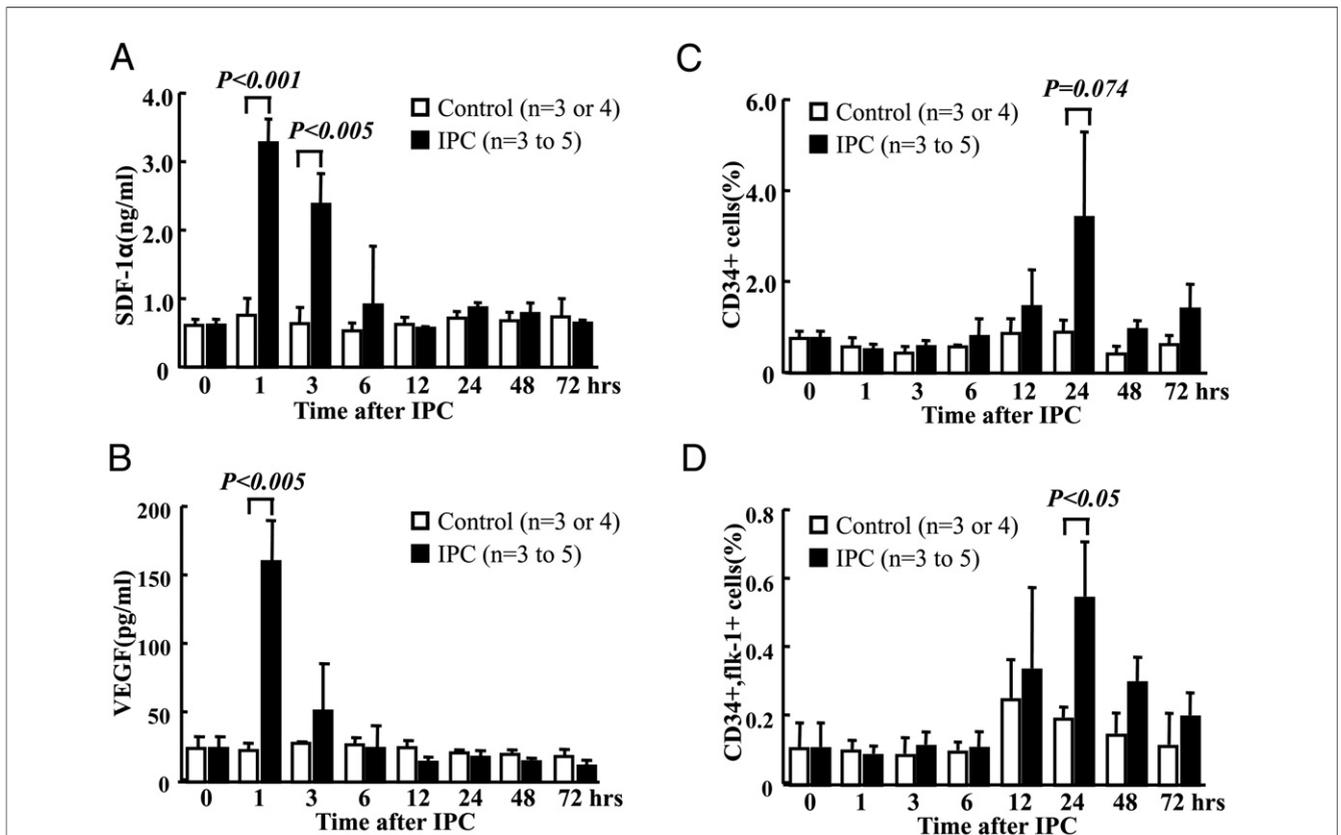


Figure 1 Kinetics of Cardioprotective Factors and Stem Cells in the Peripheral Blood After IPC

Enzyme-linked immunosorbent assay (ELISA) measurement showed remarkably increased plasma stromal cell-derived factor (SDF)-1 α (A) and vascular endothelial growth factor (VEGF) (B) 1 and 3 h after ischemic pre-conditioning (IPC), which returned to the baseline level from 6 h onward. Conversely, CD34+ cells (C) and CD34+/flk-1+ cells (D) in the peripheral blood increased from 12 h after IPC and peaked at 24 h. Data at each time point were derived from 3 to 5 mice, and ELISA was performed by a duplicative assay.

Coculture of adult cardiomyocytes and BMSCs. To confirm the cardioprotective effect of BMSCs, we cocultured adult cardiomyocytes with BMSCs as described previously (13). Adult cardiomyocytes were isolated from 13-week-old Wistar rats. Freshly isolated cardiomyocytes were placed on laminin-coated culture slides, and cocultured with or without bone marrow mononuclear cells. After 7 days of cultivation, the apoptotic cardiomyocytes were detected by TUNEL assay with Apoptosis Detection Kits (R&D Systems, Inc.).

Intervention study by blocking the recruitment of BMSCs into I/R injured heart. To confirm that the cardioprotective effect of IPC is related directly to the mobilization and recruitment of BMSCs, an intervention study was performed by blocking the recruitment of BMSCs into the I/R injury heart after IPC. The blockade of the recruitment of BMSCs was done by the antibody disruption of the SDF-1/CXCR4 axis as described previously (17), and a nonspecific action of antibody was controlled by the administration of the same dose of nonspecific immunoglobulin. Briefly, mice were given an intraperitoneal injection with 10 μ g/kg rabbit antimouse CXCR4 polyclonal antibody (Cell Science Inc., Canton, Massachusetts) or nonspecific immunoglobulin 2 h before IPC (early IPC), 22 h after IPC (late IPC), or 2 h before sham laparotomy (control). The I/R injury was induced immediately after IPC (early IPC), 24 h after IPC (late IPC), or immediately after sham laparotomy (control). All surgical procedures, cardiac function measurement, and

histological analysis were done as described in the preceding text.

Statistical analysis. All data are presented as mean \pm SD. Statistical significance between 2 groups was determined by the 2-tailed unpaired Student *t* test, and among groups it was determined by a 1-way analysis of variance followed by Bonferroni or Scheffe's post-hoc test with the StatView software (version 5.0, SAS Institute, Cary, North Carolina). Differences were considered statistically significant when $p < 0.05$.

Results

IPC increased cardioprotective factors in the early phase but enhanced mobilization of BMSCs in the late phase.

Ischemic pre-conditioning induced a 4-fold increase in plasma SDF-1 α compared with the control, 1 and 3 h after IPC ($p < 0.005$) (Fig. 1A). Similarly, the level of plasma VEGF was also increased approximately 6-fold that of the control 1 h after IPC ($p < 0.005$) (Fig. 1B). However, the SDF-1 α and VEGF had returned to almost the baseline levels 6 h after IPC and had not re-increased 12, 24, 48, or 72 h after IPC (Figs. 1A and 1B).

The CD34+ and CD34+/flk-1+ stem cells in the peripheral blood were not higher in the early phase of IPC than in the control. Conversely, IPC induced an increase of CD34+ and CD34+/flk-1+ stem cells in the peripheral blood at 12 h, and this increase peaked 24 h after IPC (Figs. 1C and 1D).

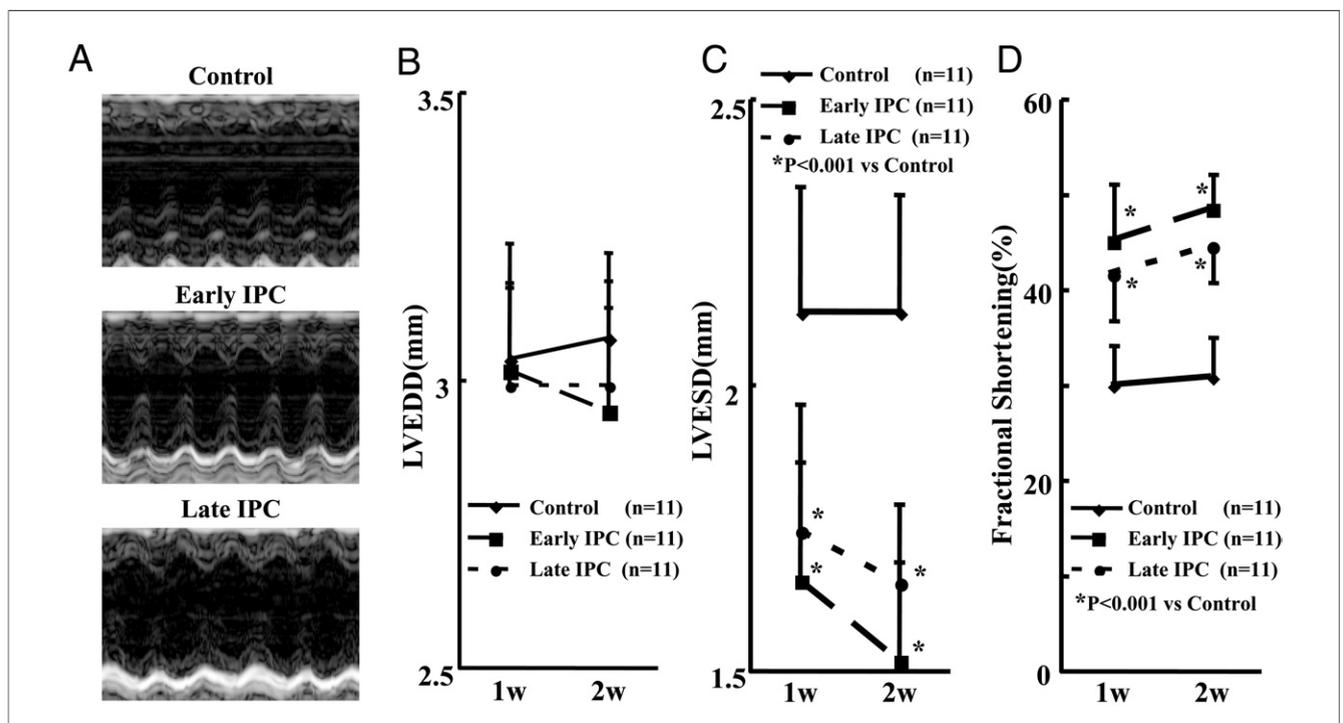


Figure 2 Echocardiographic Assessment of Cardiac Function

(A) Representative M-mode echocardiograms in each group, 2 weeks after ischemia/reperfusion (I/R) heart injury. The left ventricular end-diastolic diameters (LVEDDs) (B), left ventricular end-systolic diameters (LVESDs) (C), and left ventricular fractional shortening (D) were measured 1 and 2 weeks after I/R heart injury. IPC = ischemic pre-conditioning.

Early and late phases of IPC protected the heart against I/R injury. Echocardiography showed obviously better wall motion of the LV anterior wall in the early and late IPC groups than in the control group (Fig. 2A). Although the LVEDDs did not differ among the groups (Fig. 2B), the LVESDs were significantly lower in both the early and late IPC groups than in the control group, 1 and 2 weeks after I/R injury ($p < 0.001$) (Fig. 2C). The LVFS% was also significantly higher in the early and late IPC groups than in the control group, 1 and 2 weeks after I/R injury ($p < 0.001$) (Fig. 2D).

Histological analysis revealed that the thickness of the LV anterior wall did not differ significantly among groups (Fig. 3A). However, there was significantly less fibrosis of the LV in both the early and late IPC groups than in the control group 2 weeks after I/R injury ($p < 0.005$) (Fig. 3B). Furthermore, TUNEL staining analysis showed fewer apoptotic cells in the early and late IPC groups than in the control group 1 day after I/R injury ($p < 0.001$) (Fig. 3C). All of these findings indicated that

remote IPC could protect the heart against I/R injury in both the early and late phases.

Enhanced recruitment of BMSCs to the I/R injured heart in the late phase of IPC. Immunostaining analysis showed that the BMSCs were localized mainly in the risk area after I/R injury, although very few of the BMSCs were also observed in the area without I/R injury. By quantitative counting, we found that the early and late IPC groups had a significantly higher number of bone marrow-derived GFP+/Sca-1+ (Fig. 4A) and GFP+/c-kit+ (Fig. 4B) stem cells in the risk area of the I/R injured heart than the control group without remote IPC. Furthermore, the recruitment of GFP+/c-kit+ and GFP+/Sca-1+ BMSCs was approximately 2-fold higher in the late IPC group than in the early IPC group ($p < 0.05$) (Figs. 4A and 4B).

By quantitative flow cytometry analysis, we confirmed that the bone marrow-derived GFP+/Sca-1+ (Fig. 4C) and GFP+/c-kit+ (Fig. 4D) stem cells were also approximately 2- to 3-fold higher in the late IPC group than in the early IPC group.

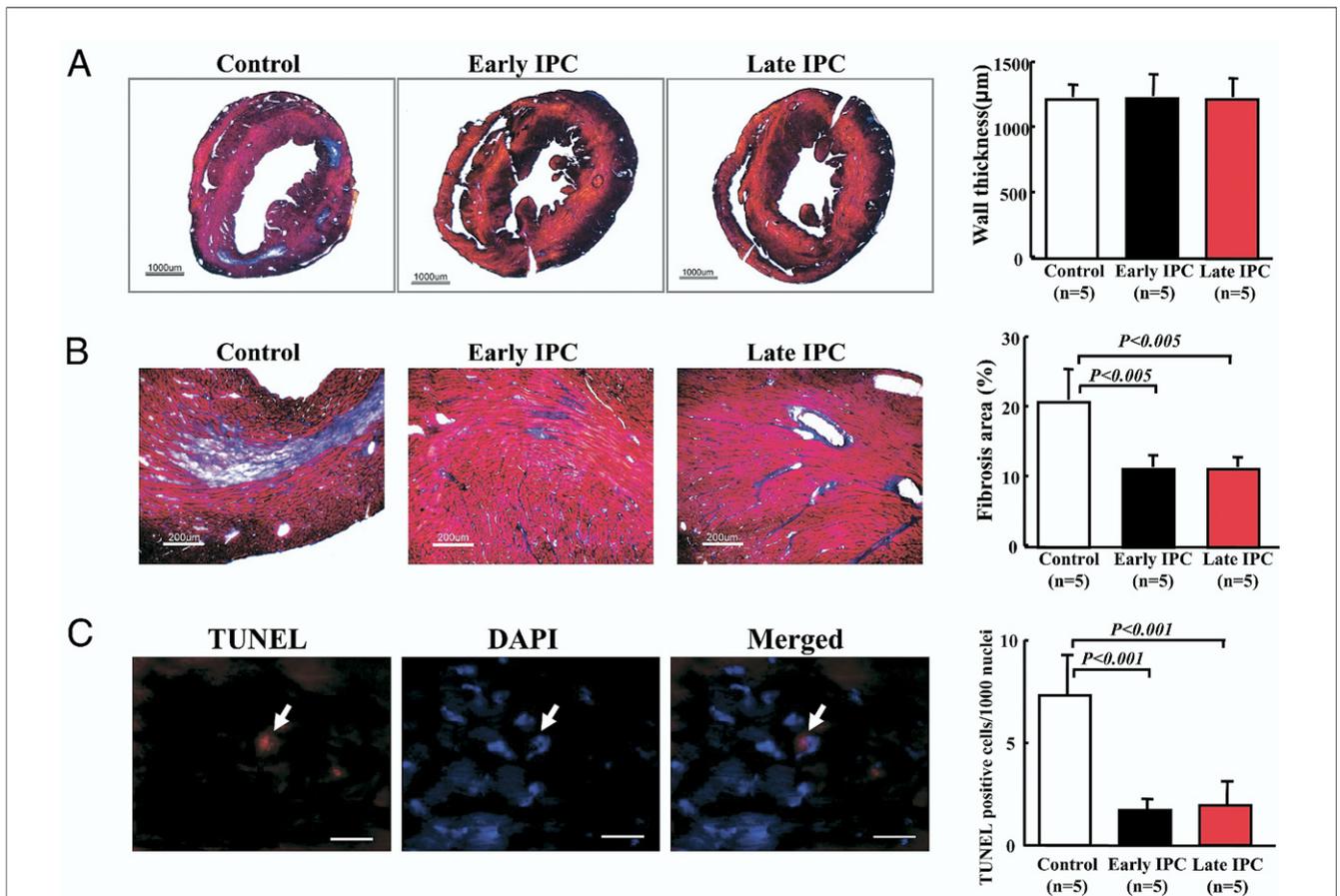
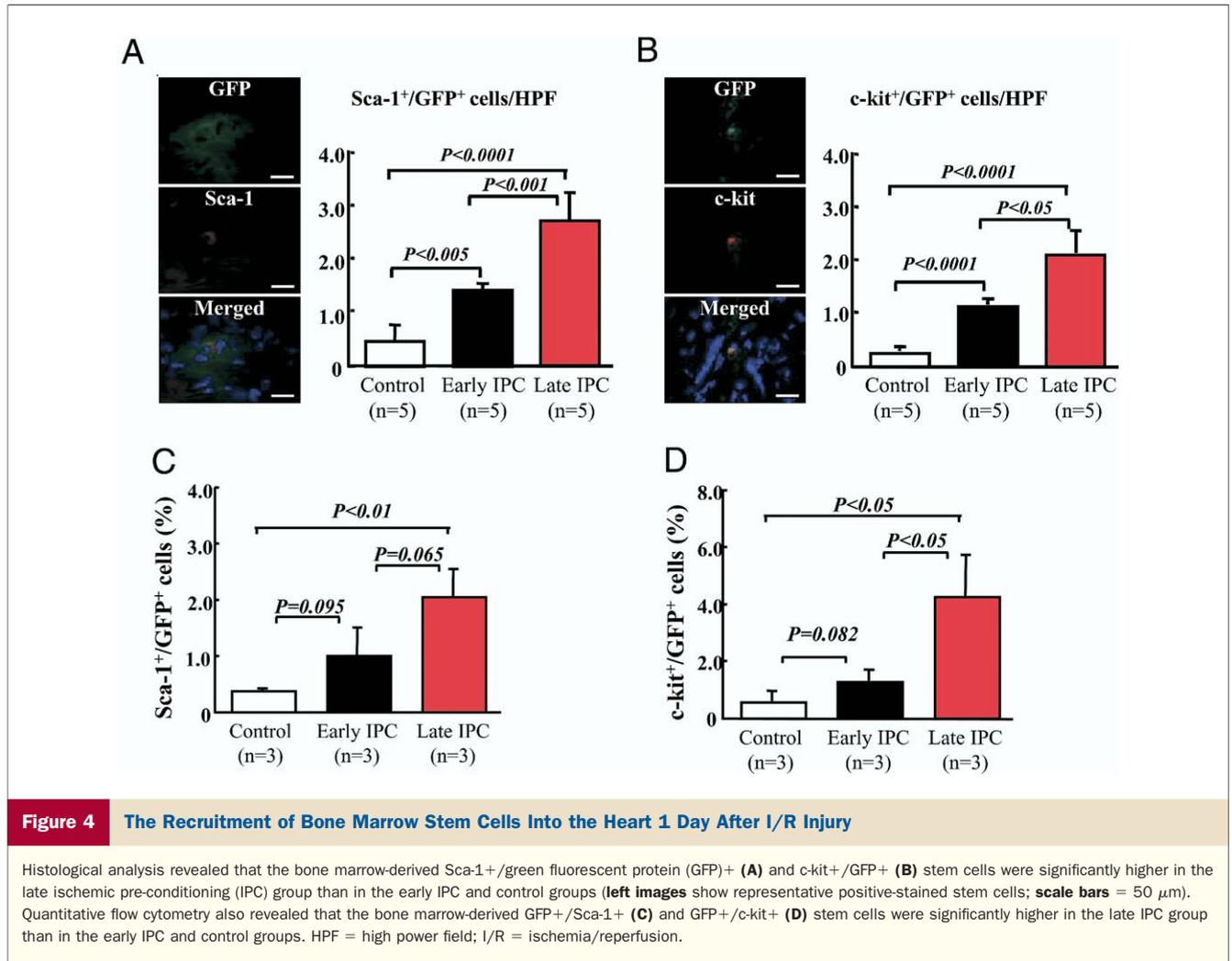


Figure 3 Histological Examinations

Although the left ventricular wall thickness did not differ among the groups (A, right), quantitative analysis revealed significantly fewer fibrotic areas in the early and late ischemic pre-conditioning (IPC) groups than in the control group (B, right) by Azan staining 2 weeks after ischemia/reperfusion injury. (C) Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assessment of apoptotic cells revealed significantly fewer apoptotic cells in the early and late IPC groups than in the control group (arrows in left images show representative TUNEL-positive apoptotic cell from the control group, scale bar = 20 µm). DAPI = 4'-6-diamidino-2-phenylindole.



Differentiation of BMSCs after recruitment in the I/R injured heart. We observed positive expression of the GFP+ cells to the endothelial-specific marker of VE-cadherin in the heart 2 weeks after I/R injury (Fig. 5A). Furthermore, we also found occasional troponin-I positive GFP+ cells in the heart 2 weeks after I/R injury (Fig. 5A). These findings indicated that BMSCs could be differentiated into myocytes and endothelial cells.

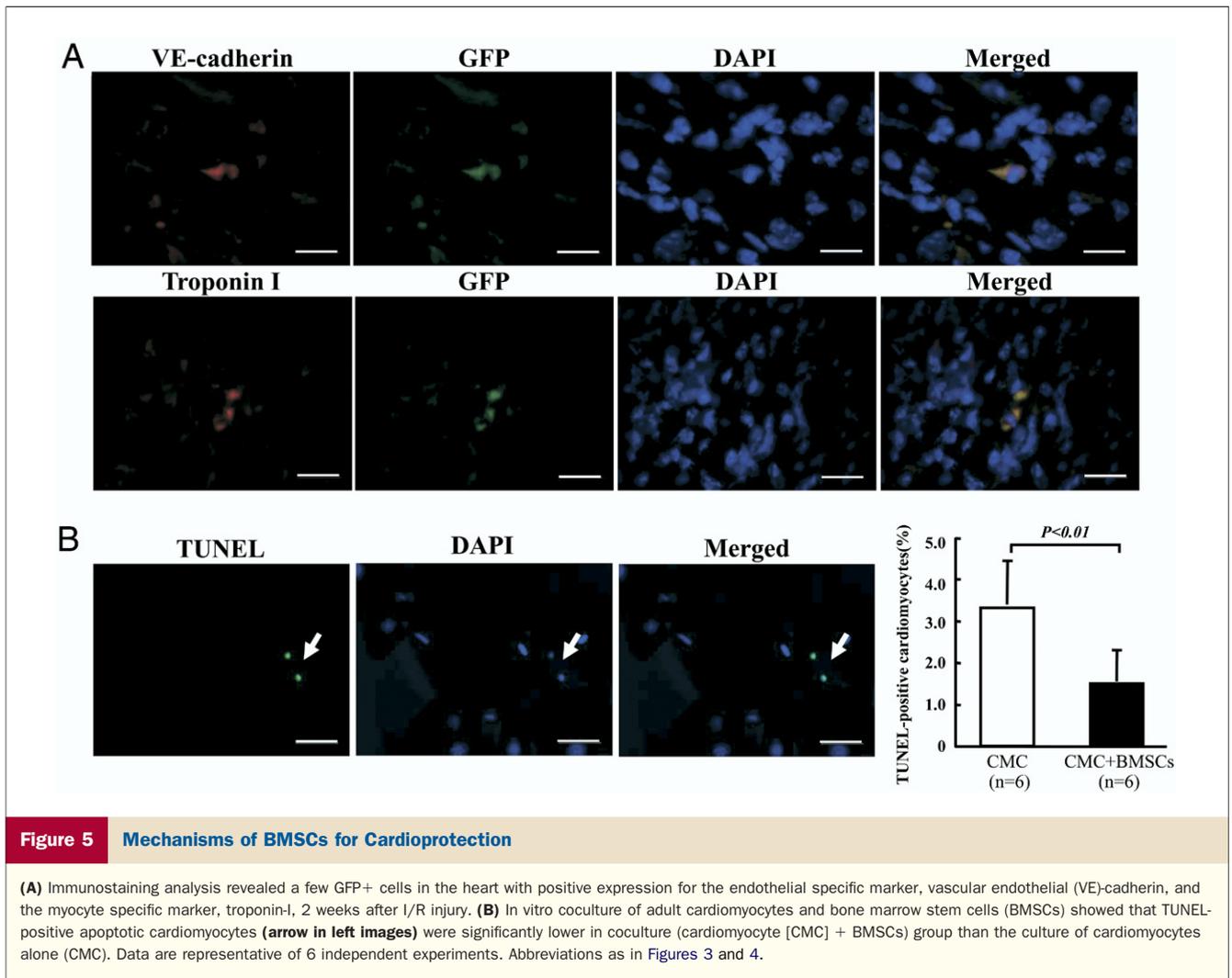
Cardioprotective effect of BMSCs through paracrine mechanisms. There were significantly fewer TUNEL-positive cardiomyocytes 7 days after coculture with BMSCs than the cultivation of cardiomyocytes only ($p < 0.01$) (Fig. 5B). This indicated that BMSCs could protect cardiomyocytes against apoptosis through paracrine mechanisms.

Cardioprotective effect was attenuated by blocking the recruitment of BMSCs in the late phase but was not in the early phases of IPC. The LVFS% was significantly lower in the mice that received anti-CXCR4 antibody injections than in those that received nonspecific immunoglobulin 1 and 2 weeks after I/R injury when the I/R heart injury was induced in the late phase of IPC (late IPC group) ($p < 0.01$) (Fig. 6A, right). However, when the I/R heart injury was

induced in the early phase of IPC (early IPC group) or sham laparotomy (control group) (Fig. 6A, left), the LVFS% was not significantly different between the injection of anti-CXCR4 antibody and nonspecific immunoglobulin. Compared with nonspecific immunoglobulin, histological analysis also showed that the administration of anti-CXCR4 antibody increased significantly the fibrotic area of the LV in the late IPC group ($p < 0.01$) (Fig. 6B), but it did not change in the early IPC and control groups 2 weeks after I/R injury (Fig. 6B). Furthermore, quantitative counting showed that the injection of anti-CXCR4 antibody significantly decreased the GFP+/Sca-1+ and GFP+/c-kit+ stem cells ($p < 0.01$) (Figs. 6C and 6D) in the heart 24 h after I/R injury in both early and late IPC groups. All data suggested that the inhibition of the recruitment of BMSCs by antibody disruption attenuated specifically the cardioprotective effects in the late phase of IPC but not in the early phase of IPC.

Discussion

Rapid developments in stem cell research have revealed that BMSCs can protect cardiomyocytes against apoptosis and



repair the damaged heart (13–15,21–23). It is well known that ischemic stimulation and many IPC-induced factors, such as VEGF, erythropoietin, and SDF-1 α , will improve the mobilization of BMSCs into peripheral blood (9,24,25). Several recent investigations have also demonstrated an increase of circulating BMSCs after IPC (11,12). More interestingly, in clinical stem cell therapy, a technique of repetitive balloon dilations (brief exposure to ischemia) that is used for intracoronary cell delivery has been found to facilitate the transfer of BMSCs (14,15). All of these suggested a potential relationship between IPC and BMSCs. Thus, we investigated whether the beneficial effects of IPC are related to the mobilization and the recruitment of BMSCs, especially in the late phase.

By monitoring the kinetics of stem cells and various cardioprotective factors, we observed a distinct increase of stem cells in the peripheral blood from 12 to 72 h after IPC, but SDF-1 α and VEGF in plasma increased 1 and 3 h after IPC. The phase-specific kinetics of the stem cells and cardioprotective factors suggested the release of various factors would account for most of the cardioprotection in the early phase of IPC, but the delayed cardioprotection of

IPC might be related to the mobilization and recruitment of stem cells rather than the up-regulation of some cardioprotective factors.

To further examine our hypothesis, we conducted in vivo studies by inducing I/R injury in the heart at different timings after IPC. Remote IPC by transient skeletal muscle ischemia did not affect the heart directly, which enabled us to investigate the beneficial effects of IPC on the heart and the mechanisms responsible at different timings. As expected, both the early and late phases of IPC afforded cardioprotection against I/R injury. Interestingly, the recruitment of BMSCs in the hearts was approximately 2-fold higher after the induction of I/R injury in the late phase than in the early phase of IPC. This indicated that the induction of I/R heart injury at different times of IPC would result in different grades of recruitment of BMSCs. Our data showed that the mobilization of BMSCs into circulating blood peaked 24 h after IPC. This provided a reasonable explanation for why the recruitment of BMSCs was enhanced in the hearts subjected to I/R injury in the late phase of IPC. These findings also phenomenally supported a relation-

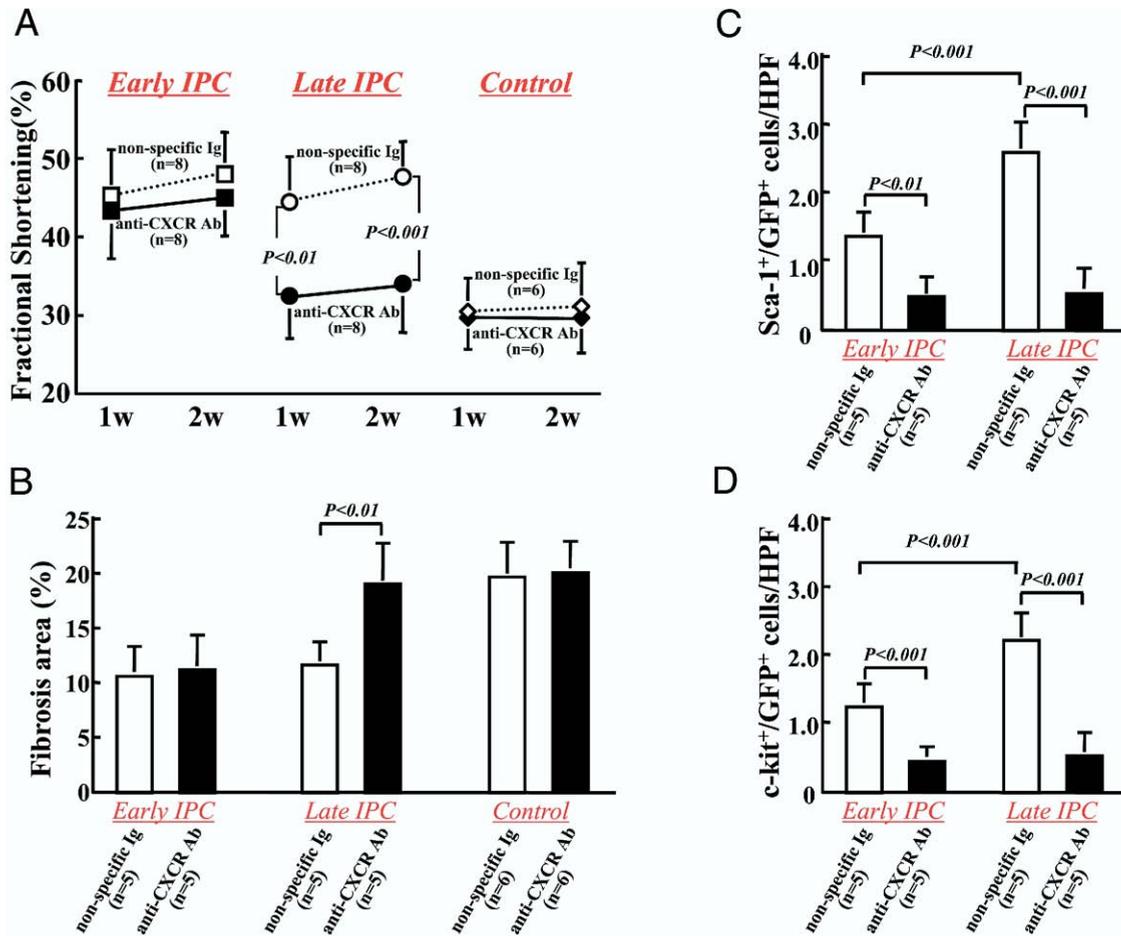


Figure 6 Blocking the Recruitment of BMSCs into the I/R Injured Heart by Antibody Disruption After IPC

(A) Compared with the injection of nonspecific immunoglobulin, the administration of anti-CXCR4 antibody decreased significantly the left ventricular fractional shortening (LVFS%) in the mice that had the I/R heart injury induced 24 h after IPC (late IPC, middle) but did not change significantly the LVFS% in the mice that had the I/R heart injury induced immediately after IPC (early IPC, left) or sham laparotomy (control, right), 1 and 2 weeks after I/R injury. (B) Histological analysis showed that the administration of anti-CXCR4 antibody significantly increased the fibrotic area only in the late IPC group but not in the early IPC group or control group 2 weeks after I/R injury. (C and D) Quantitative histological analysis showed that the GFP+/Sca-1+ stem cells (C) and GFP+/c-kit+ stem cells (D) in the heart were significantly lower in the mice that received anti-CXCR4 antibody injection than in the mice that received nonspecific immunoglobulin 24 h after I/R injury. Abbreviations as in Figures 2 and 4.

ship between the recruitment of BMSCs and the cardioprotection in the late phase of IPC.

It has been reported that the reduction in myocardial metabolism and energy consumption also contributes to cardioprotection in the late phase of classical IPC (26). However, the metabolic modification should not be the main mechanism in the late phase of IPC in this study, because we used a remote IPC model without direct distribution of the heart before I/R heart injury. Otherwise, we cannot deny that the up-regulation of some other molecules would also contribute to the delayed cardioprotection of IPC, because we did not investigate the changes of other factors extensively, especially in the I/R injured heart.

To determine a causative relationship between the recruitment of BMSCs and the cardioprotection of late phase IPC, we examined the cardioprotective effect of IPC by blocking the recruitment of BMSCs. We found that the

blockade of the recruitment of BMSCs eliminated almost completely the cardioprotective effects of IPC at the late phase. However, the cardiac function was not significantly changed by the administration of anti-CXCR4 antibody either in the early phase of IPC or after sham laparotomy. This strongly suggests that the recruitment of BMSCs into the injured heart played an important role for cardioprotection in the late phase but not in the early phase of IPC.

The mechanisms of BMSCs for cardioprotection in IPC remain unclear. However, previous studies have reported that BMSCs can protect and repair a damaged heart by inducing angiogenesis (14,15,22), differentiating into endothelial cells and myocytes to regenerate new vessel and myocardium (22), as well as other paracrine mechanisms (13,22). However, the differentiation of BMSCs into endothelial cells was relatively rare and slow, and the differentiation of BMSCs into cardiomyocytes is still controversial (27). Therefore, the IPC-induced

mobilization and recruitment of BMSCs is more likely to protect the heart against I/R injury by the production of various cardioprotective factors, including VEGF, platelet-derived growth factor, and insulin-like growth factor-1 (13,22), rather than repair by the differentiation and maturation of BMSCs. Our data of coculture of cardiomyocytes with BMSCs also supported the paracrine mechanisms.

In this study, we used healthy mice and did not estimate the quality of BMSCs, including their migratory and proliferation potential. However, diabetes mellitus, hyperlipidemia, aging, and many other factors have been found to contribute to a functional impairment of BMSCs (28). Therefore, it will be very interesting to investigate whether the cardioprotective effect of IPC is also an individual difference due to different capacities of mobilization and recruitment of BMSCs after IPC.

Conclusions

We found that IPC can induce the mobilization of BMSCs in the late phase. These mobilized BMSCs were recruited to the damaged heart and contributed to protecting the heart against I/R injury. Our results demonstrate for the first time that IPC enhances the mobilization and recruitment of BMSCs in the late phase to protect the heart against I/R injury, which provides new insight into the mechanism of late-phase IPC.

Reprint requests and correspondence: Dr. Tao-Sheng Li, Department of Surgery and Clinical Science, Yamaguchi University, Graduate School of Medicine, 1-1-1 Minami Kogushi, Ube, Yamaguchi 755-8505, Japan. E-mail: litaoshe@yamaguchi-u.ac.jp.

REFERENCES

1. Murry CE, Jennings RB, Reimer KA. Pre-conditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 1986;74:1124–36.
2. Przyklenk K, Bauer B, Ovize M, Kloner RA, Whittaker P. Regional ischemic pre-conditioning protects remote virgin myocardium from subsequent sustained coronary occlusion. *Circulation* 1993;87:893–9.
3. Kuzuya T, Hoshida S, Yamashita N, et al. Delayed effects of sublethal ischemia on the acquisition of tolerance to ischemia. *Circ Res* 1993;72:1293–9.
4. Tang XL, Qiu Y, Park SW, Sun JZ, Kalya A, Bolli R. Time course of late pre-conditioning against myocardial stunning in conscious pigs. *Circ Res* 1996;79:424–34.
5. Murphy E, Steenbergen C. Mechanisms underlying acute protection from cardiac ischemia-reperfusion injury. *Physiol Rev* 2008;88:581–609.
6. Stein AB, Bolli R, Guo Y, et al. The late phase of ischemic pre-conditioning induces a prosurvival genetic program that results in marked attenuation of apoptosis. *J Mol Cell Cardiol* 2007;42:1075–85.
7. Xuan YT, Guo Y, Han H, Zhu Y, Bolli R. An essential role of the JAK-STAT pathway in ischemic pre-conditioning. *Proc Natl Acad Sci U S A* 2001;98:9050–5.
8. Xuan YT, Tang XL, Banerjee S, et al. Nuclear factor-kappa B plays an essential role in the late phase of ischemic pre-conditioning in conscious rabbits. *Circ Res* 1999;84:1095–109.
9. Cai Z, Manalo DJ, Wei G, et al. Hearts from rodents exposed to intermittent hypoxia or erythropoietin are protected against ischemia-reperfusion injury. *Circulation* 2003;108:79–85.
10. Kawata H, Yoshida K, Kawamoto A, et al. Ischemic pre-conditioning upregulates vascular endothelial growth factor mRNA expression and neovascularization via nuclear translocation of protein kinase C epsilon in the rat ischemic myocardium. *Circ Res* 2001;88:696–704.
11. Li M, Nishimura H, Iwakura A, et al. Endothelial progenitor cells are rapidly recruited to myocardium and mediate protective effect of ischemic pre-conditioning via imported nitric oxide synthase activity. *Circulation* 2005;111:1114–20.
12. Patschan D, Krupinca K, Patschan S, Zhang Z, Hamby C, Goligorsky MS. Dynamics of mobilization and homing of endothelial progenitor cells after acute renal ischemia: modulation by ischemic pre-conditioning. *Am J Physiol Renal Physiol* 2006;291:176–85.
13. Takahashi M, Li TS, Suzuki R, et al. Cytokines produced by bone marrow cells can contribute to functional improvement of the infarcted heart by protecting cardiomyocytes from ischemic injury. *Am J Physiol Heart Circ Physiol* 2006;291:H886–93.
14. Strauer BE, Brehm M, Zeus T, et al. Regeneration of human infarcted heart muscle by intracoronary autologous bone marrow cell transplantation in chronic coronary artery disease: the IACT study. *J Am Coll Cardiol* 2005;46:1651–8.
15. Chen SL, Fang WW, Ye F, et al. Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction. *Am J Cardiol* 2004;94:92–5.
16. Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y. “Green mice” as a source of ubiquitous green cells. *FEBS Lett* 1997;407:313–9.
17. Murakami J, Li TS, Ueda K, Tanaka T, Hamano K. Inhibition of accelerated tumor growth by blocking the recruitment of mobilized endothelial progenitor cells after chemotherapy. *Int J Cancer* 2008;124:1685–92.
18. Weinbrenner C, Schulze F, Sárváry L, Strasser RH. Remote pre-conditioning by infrarenal aortic occlusion is operative via delta1-opioid receptors and free radicals in vivo in the rat heart. *Cardiovasc Res* 2004;61:591–9.
19. Li TS, Mikamo A, Takahashi M, et al. Comparison of cell therapy and cytokine therapy for functional repair in ischemic and nonischemic heart failure. *Cell Transplant* 2007;16:365–74.
20. Li TS, Suzuki R, Ueda K, Murata T, Hamano K. Analysis of the origin and population dynamics of cardiac progenitor cells in a donor heart model. *Stem Cells* 2007;25:911–7.
21. Orlic D, Kajstura J, Chimenti S, et al. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. *Proc Natl Acad Sci U S A* 2001;98:10344–9.
22. Kinnaird T, Stabile E, Burnett MS, et al. Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. *Circ Res* 2004;94:678–85.
23. Jackson KA, Majka SM, Wang H, et al. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. *J Clin Invest* 2001;107:1395–402.
24. Hu X, Dai S, Wu WJ, et al. Stromal cell-derived factor-1 α confers protection against myocardial ischemia/reperfusion injury. *Circulation* 2007;116:654–63.
25. Dai Y, Xu M, Wang Y, Pasha Z, Li T, Ashraf M. HIF-1 α induced-VEGF overexpression in bone marrow stem cells protects cardiomyocytes against ischemia. *J Mol Cell Cardiol* 2007;42:1036–44.
26. Vuorinen K, Ylitalo K, Peuhkurinen K, Raatikainen P, Ala-Rämi A, Hassinen IE. Mechanisms of ischemic pre-conditioning in rat myocardium. Roles of adenosine, cellular energy state, and mitochondrial F1F0-ATPase. *Circulation* 1995;91:2810–8.
27. Alvarez-Dolado M, Pardal R, Garcia-Verdugo JM, et al. Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. *Nature* 2003;425:968–73.
28. Kissel CK, Lehmann R, Assmus B, et al. Selective functional exhaustion of hematopoietic progenitor cells in the bone marrow of patients with postinfarction heart failure. *J Am Coll Cardiol* 2007;49:2341–9.

Key Words: bone marrow stem cell ■ ischemic pre-conditioning ■ late phase ■ mobilization ■ recruitment.