Regulation of Cardiac Regeneration by ACE Inhibition Following Donor Heart Myocardial Infarction After Heterotopic Transplantation in Tg Mice

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Background  Experimental and clinical evidence have recently shown that pluripotent stem cells can be mobilized using granulocyte-colony stimulating factor (GCSF) and may enhance myocardial regeneration after acute myocardial infarction (MI). The present study investigated the pharmacological role of angiotensin-converting enzyme inhibition on cardiac regeneration after MI using a mouse model of heterotopic cardiac transplantation and coronary ligation.

Methods and Results  Isogenic heterotopic cardiac transplantations and simultaneous coronary ligations were performed in green fluorescent protein (GFP) mice to produce MI in the donor heart. Five mice in the ligation group were treated with oral perindopril (PE) after the operation. Three mice in the ligation group were treated with subcutaneous GCSF and 4 angiotensin II type 1a receptor knockout (AT1aRKO) mice were used as well. At 60 days after the operation, the maximum GFP-positive cell counts in the GCSF group were significantly higher than in the other 4 groups. The maximum GFP-positive cell counts in both the AT1aRKO and ligation & PE groups were significantly higher than in the sham and ligation groups.

Conclusions  Pharmacological modification for cardiac regeneration may provide an alternative treatment for subsequent cardiac remodeling in the late phase of MI. (Circ J 2008; 72: 793–799)

Key Words: Cardiac transplantation; Myocardial infarction; Regeneration

Postischemic heart failure has become the leading cause of death in developed countries. Hundreds of thousands of new cases are diagnosed each year, and despite a large battery of pharmacological agents, heart transplantation remains the ultimate therapy for patients with end-stage heart failure. However, the request for organs far exceeds the number of potential donors. As an alternative approach, the regeneration of the myocardium via controlled differentiation of cardiomyocyte progenitor cells is receiving much attention. Recent research has revealed that, although a small number of cardiomyocytes do undergo cell division immediately after an myocardial infarction (MI), their contribution is not sufficient to prevent heart failure. Heart transplantation is traditionally performed to treat intractable severe heart failure secondary to dilated and hypertrophic cardiomyopathy, but its use is restricted by a shortage of donors. The use of pluripotent stem cells to regenerate damaged heart tissue is being advocated as a new treatment for heart failure secondary to heart disease or severe MI. Angiotensin-converting enzyme inhibitors (ACEI) and angiotensin receptor blockers were expected to prevent cardiac remodeling, as well as to reduce morbidity and mortality in patients with MI. The VALIANT trial demonstrated that valsartan was as effective as captopril in patients who suffered from MI. It has been reported that candesartan, cilexetil, and cilazapril all improved systolic and diastolic function, and prevented ventricular remodeling after MI in rats. Granulocyte-colony stimulating factor (GCSF) is a cytokine that mobilizes CD34+ EPC from the bone marrow into the peripheral blood. Clinically, GCSF has been used for the collection of stem cells used in allogeneic or syngeneic peripheral blood stem cell transplantation. Notably, GCSF appears to induce vascular and myocardial tissue regeneration and accelerate the healing process in the infarcted myocardium of experimental animals. To examine different kinds of pharmacological effects on cardiac regeneration after MI, we performed isogenic heterotopic cardiac transplantation and simultaneous coronary ligation in mice in the present study and examined green fluorescent protein (GFP)-positive cells in the donor heart.

Methods  The experimental protocols used in the present study were approved by the Ethics Committee for Animal Experimentation at Yamaguchi University School of Medicine, and carried out according to the Guidelines for Animal Experimentation at Yamaguchi University School of Medicine, and Law No. 105 and Notification No. 6 of the Japanese Government. The study protocol was approved by the Institutional Animal Care and Use Committee of...
Yamaguchi University School of Medicine (23-034), and followed the guidelines of the American Heart Association.

Animals
Ten-week old, inbred, certified, C57BL/6 male mice (Japan SLC, Shizuoka, Japan), weighing approximately 20 g, were housed individually under a controlled room temperature and fed laboratory chow. GFP mice were obtained from Dr. Okabe (Osaka University) and used for all recipients. Three angiotensin II type1a receptor knockout (AT1aRKO) mice were supplied by Tanabe pharmaceutical company and used as donors in the present study.

Heterotopic Cardiac Transplant-Coronary Ligation
Isogenic heterotopic cardiac transplantation was performed according to the method of Corry. To induce MI in the transplanted heart, proximal left anterior descending coronary artery ligation in the donor heart was performed immediately after transplantation during the same surgical intervention. In the present study, GFP mice were used as recipients (Fig 1). Myocardial ischemia was confirmed by regional cyanosis. Technical failures observed within 72 h were excluded from evaluation. As a sham operation, non-ligated hearts were transplanted into the same strain of mice. The viability of the donor heart was assessed based on the beating of the transplanted heart. To examine the effect of angiotensin-converting enzyme (ACE) inhibition, six mice in the ligation group were treated postoperatively with perindopril. The perindopril was added to their drinking water at a dose of 1 mg·kg−1·day−1. Three mice in the ligation group were treated with GCSF 3000 μg·kg−1·day−1×10 days s.c. after the operation. No immunosuppressive agents were administered.

Morphometry and Histopathological Study
On day 60 post surgery, both body and heart weights were measured. The donor hearts were cut in half transversely. After dehydration in an ethanol gradient (70–100% each 90 min), samples were embedded in paraffin for 180 min and sectioned into of 5 μm thick slices. To detect myosin and α-actinin expression in GFP-positive cells, myocytes were stained using mouse anti-α-actinin antibody and anti-myosin antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA), respectively, as primary antibodies and FITC-goat primary antibody to GFP (Santa Cruz Biotechnology, Inc), then goat anti-rabbit IgG. Slides of the stained tissues were prepared and analyzed using fluorescence and confocal LASER microscopy (LSM5 Pascal, Carl Zeiss, Germany). Maximum numbers of GFP-positive cells were counted in 20 serial sections by 2 investigators who had no knowledge of the data.

Quantification of Cardiac Cell Fusion
To quantify cell fusion, sex-mismatch transplantation was performed followed by fluorescence in situ hybridization (FISH) staining for the X and Y chromosomes. The biotin labeled mouse Y-chromosome-painting probe (Cambio Ltd: Cambridge, UK) and the bacterial artificial chromosome (BAC)-derived probe targeting the telomeric region of the mouse X chromosome were used for the FISH analysis. BAC clone RPCI-23 202H24 (Invitrogen, USA) was labeled with digoxigenin using the Nick Translation Kit (Roche, Switzerland) according to the manufacturer’s protocol. Dual-color FISH analyses were carried out by Chromosome Science Labo Inc (Sapporo, Japan). The 4 μm-thick, formalin-fixed and paraffin-embedded sections were deparaffinized 5 times with xylene, rehydrated 3 times with ethanol and rinsed twice with PBS. Target retrievals were carried out by heating in a microwave oven for 10 min in 2×SSC, followed by protease digestion with 0.1% pepsin in 0.1N HCl at 37°C for 2.5 min. After washing with PBS, the slides were dehydrated with alcohol and air dried. Ten microlitres of the probe mixture was applied to each slide and covered with a coverslip, followed by simultaneous denaturation at 90°C for 15 min. The slides were then incubated at 37°C overnight in a humidified chamber. After hybridization, the coverslips were removed and the slides were washed for 20 min with 50% formamide in 2×SSC at 37°C and 15 min with 1×SSC at room temperature. The slides were then blocked with a blocking solution (10% goat serum and 3% BSA in 4×SSC) for 30 min at 37°C, followed by antibody treatment with streptavidin-Cy3 (1/300) and anti-digoxigenin-Cy5 (1/200) in blocking solution at 37°C for 30 min. The slides were washed twice with 0.1% Nonidet P-40 in 4×SSC and once with 4×SSC. Nuclei were counterstained with DAPI and the slides were mounted with antifade solution. The FISH images were captured and analyzed using the Leica CW4000 system.

Statistical Analysis
Data are expressed as the mean±SEM. Differences between the groups were assessed with 1-way ANOVA and FISHER post-hoc testing. A p value <0.05 was considered statistically significant.

Results
Histological Analysis
The surgical success rate was 95%. Sixty days after the operation, scar formations were observed in the left ventricular (LV) free wall in all groups with coronary ligation (Fig 2). All mice that had non-beating donor hearts were excluded from the study. There was no significant difference in body weight among the 5 groups after the operation. The donor heart weights in the ligation, GCSF and AT1aRKO groups were significantly increased compared with the sham group, whereas the donor heart weight in the ligation and perindopril group showed a tendency to in-

Fig 1. Heterotopic transplant-coronary ligation in mice. (A) An anatomic scheme of cardiac transplantation and simultaneous coronary artery ligation in green fluorescent protein (GFP) recipient mice. (B) Sixty days after the operation, we examined the expression of GFP-positive cells that were located in the GFP-negative wild donor heart.
crease compared with the sham group. In contrast, the donor heart weight in the ligation & PE group was significantly decreased compared with the ligation and GCSF groups (p<0.05). There was no significant difference between the ligation & PE and AT1aRKO groups (Fig 3).

Examination With Laser Confocal Microscopy

GFP-positive cells were mainly found in clusters in the epicardium of the donor heart. The maximum GFP-positive cell count in the GCSF group was significantly higher than in the other 4 groups. The maximum GFP-positive cell count in both the AT1aRKO and ligation & PE groups were significantly higher than in the sham and ligation groups. There was no significant difference between the ligation & PE and AT1aRKO groups (Fig 4).

Immunohistochemistry

GFP-positive cells were mainly localized in the epicardium of the donor heart. We examined the GFP-positive cells by immunostaining with cardiac-specific proteins (myosin and ß-actinin; cardiac contractile proteins) (Fig 5).

FISH Analysis for Cell Fusion

To confirm both cardiac cell fusion and differentiation, histological sections of the left ventricle in gender-mismatched mice were evaluated in both the sham and ligated groups. Yellow and red dots in Fig 6 indicate Y and X chromosomes, respectively. The Y chromosome was located eccentrically within the nuclei of chimeric cardiomyocytes, and chromosomal ploidy analysis was used to obtain evidence of cell fusion between donor and recipient-derived cells. We examined all nuclei that had Y chromosomes (yellow color) in 20 serial sections from the donor hearts. The presence of more than 2 X chromosomes (red color) for each Y chromosome in the nucleus was counted as a cardiac cell fusion. The ratio of cell fusion was up to 2.0% in all groups, there was no significant difference among all groups (Fig 6).
Fig 5. Immunohistochemical assessment in the donor heart after the operation. We also confirmed that these green fluorescent protein (GFP)-positive cells also express cardiac specific myosin (Upper panel), and \( \alpha \)-actinin (Lower panel) by immunological double staining.
Discussion

MI induces global changes in ventricular architecture that are collectively referred to as "post-MI remodeling". Recently, it was suggested that bone marrow-derived precursors can give rise to myocytes that contribute to the repair, remodeling and lesion formation of heart tissue under certain circumstances. Although it was previously thought that irreversibly injured cardiac tissue was replaced only by an inert scar, recent evidence suggests that some injured cells may be replaced by viable cells. Recent studies suggest that cytokines released during MI can set the stage for subsequent angiogenesis. In a post-infarction model, expression of the isoform, VEGF120, was found at days 1 and 4 after MI, whereas isoform VEGF160 and VEGF188, along with expression of TNF-α and iNOS, were noted for a much longer period of time. These findings suggest that angiogenic factors are released at the same time as proinflammatory cytokines to mediate vascular repair. Macrophages may also participate in angiogenesis; a macrophage-derived peptide, PR39, inhibited the ubiquitin-protease-dependent degradation of hypoxia-inducible factor-1α protein, resulting in accelerated formation of vascular structures in vitro and increased myocardial vasculature in intact mice.

Previously, we developed a cardiac transplant-coronary ligation model in rats that allowed quantification of indices of cardiac remodeling in the absence of preload and afterload. The model was used to evaluate the local and remote effects of angiotensin II and tissue necrotizing factor on cardiac remodeling. In the present study, to quantify both cardiac remodeling and regeneration, we applied this model. In the present study, cardiac remodeling was not suppressed. This result is similar to the recent FIRSTLINE-AMI trial that showed there was no significant improvement of LV remodeling after GCSF stimulation in patients with acute MI. The possibility that infused inflammatory cells and platelets could aggregate and disturb the microcirculation was a major concern with GCSF-based stem cell therapy. These results indicated that ACE inhibition might have pleiotropic effects beyond cardiac regeneration, which was mainly induced by GCSF after MI. Recently, Yoshiyama et al reported that treatment with an ACEI in AT1αKO mice after MI has a beneficial effect on cardiac remodeling that was not mediated through AT1 receptors. Although total GFP-positive cell number increased the most in the GCSF group, cardiac remodeling after MI was not suppressed in our model. This result is similar to the recent FIRSTLINE-AMI trial that showed there was no significant improvement of LV remodeling after GCSF stimulation in patients with acute MI. The possibility that infused inflammatory cells and platelets could aggregate and disturb the microcirculation was a major concern with GCSF-based stem cell therapy. These results indicated that ACE inhibition might have pleiotropic effects beyond cardiac regeneration, which was mainly induced by GCSF after MI. Recently, Yoshiyama et al reported that treatment with an ACEI in AT1αKO mice after MI has a beneficial effect on cardiac remodeling that was not mediated through AT1 receptors. Although total GFP-positive cell numbers were significantly increased in the AT1αKO group in the present study, cardiac remodeling was not suppressed. These findings suggest that ACE inhibition prevents LV remodeling after MI by mechanisms other than inhibition.
of AT1 receptor mediated effects. Increased kinin activation resulting from ACE inhibition, which is not dependent on an AT1 receptor mediated mechanism, might attenuate structural remodeling of the infarcted donor heart. The potential anti-remodeling action of bradykinin may relate to increased nitric oxide synthesis or an effect on prostaglandin metabolism. The MAGIC randomized clinical trial, which used GCSF therapy with intracoronary infusion of peripheral blood stem cells, stopped enrolment due to aggravation of restenosis. After 2 years of follow-up, cell infusion resulted in an increase of binary restenosis (50% vs 30%, p>0.05) and a greater late loss of minimal luminal diameter (p<0.05) at 6 months of follow-up, compared to controls. These clinical results are compatible with our experimental data. At least in the clinical setting, cardiac regeneration targeting the re-duction of remodeling after MI might not be an efficient strategy.

The concept of cardiomyocyte transplantation has been advocated since the late 1990s. Fetal or neonatal rat cardiomyocytes have been successfully transplanted into the hearts of adult rats and transplanted cells remain viable for relatively long time periods, forming gap junctions with surrounding recipient cells. Prior to the transplantation of pluripotent ES cell-derived cardiomyocytes into the heart, undifferentiated cells that differentiate into other cell types must be eliminated. Methods that use a cell sorter or drug-resistant genes to collect ES cell-derived cardiomyocytes have been reported. These include methods that specifically label cardiomyocytes by introducing agents such as GFP with a cardiomyocyte-specific promoter into ES cells. Many animal experiments have documented that adult stem cells can differentiate into other lineages. Further clinical and pharmacological studies are required to regulate cardiac regeneration after MI and the development of subsequent remodeling.

Study Limitations

In our model, the donor heart was placed in parallel with the recipient circulation, so that the donor heart continued to beat without any preload or afterload. The greatest difference between a conventional MI model and our model was not ischemia of the donor heart, but rather the loading conditions. Even the sham animals showed cardiac atrophy after transplantation, despite the fact that the donor hearts with coronary ligation had pronounced cardiac dilatation. Moreover, we could not identify the exact origin of the regeneration cells in this experiment because we used the hole approach to establish the recipient circulation, so that the donor heart continued to beat, despite the fact that the donor hearts with coronary ligation had pronounced cardiac dilatation. Moreover, we could not identify the exact origin of the regeneration cells in this experiment because we used the hole approach to establish the recipient circulation, so that the donor heart continued to beat, despite the fact that the donor hearts with coronary ligation had pronounced cardiac dilatation. Moreover, we could not identify the exact origin of the regeneration cells in this experiment because we used the hole approach to establish the recipient circulation, so that the donor heart continued to beat, despite the fact that the donor hearts with coronary ligation had pronounced cardiac dilatation.

