Kallikrein Gene Delivery Improves Cardiac Reserve and Attenuates Remodeling After Myocardial Infarction

Jun Agata, Lee Chao, Julie Chao

Abstract—In this study, we used the somatic gene delivery approach to explore the role of the kallikrein-kinin system (KKS) in cardiac remodeling and apoptosis after myocardial infarction (MI). Rats were subjected to coronary artery ligation to induce MI, and adenovirus carrying the human tissue kallikrein or luciferase gene was injected into the tail vein at 1 week after surgery. Cardiac output gradually decreased from 2 to 6 weeks after MI, whereas delivery of the kallikrein gene prevented this decrease. Cardiac responses to dobutamine-induced stress were improved in rats receiving kallikrein gene as compared with rats receiving control virus at 6 weeks after MI. Kallikrein significantly improved cardiac remodeling by decreasing collagen density, cardiomyocyte size, and left ventricular internal perimeter and increasing capillary density in the heart at 6 weeks after MI. Kallikrein gene transfer attenuated myocardial apoptosis, which was positively correlated with remodeling parameters in the heart at 2 weeks after MI. Endothelial dysfunction, characterized by increased vascular resistance, decreased left ventricular blood flow, and decreased cardiac nitric oxide levels, existed in remodeled hearts at 2 weeks after MI, whereas kallikrein gene transfer improved these parameters. Kallikrein gene delivery improved cell survival parameters as shown by increased phospho-Akt and reduced caspase-3 activation at 2 weeks after MI. This study indicates that the kallikrein-kinin system plays an important role in preventing the progression of heart failure by attenuating cardiac hypertrophy and fibrosis, improving endothelial function, and inhibiting myocardial apoptosis through the Akt-mediated signaling pathway. (Hypertension. 2002;40:653-659.)

Key Words: myocardial infarction • remodeling • kallikrein-kinin systems • genes • apoptosis

Angiotensin-converting enzyme inhibition was first shown to improve the survival of coronary ligation–induced myocardial infarction (MI) in a rat model. Treatment of the ACE inhibitor enalapril increased survival in rats with congestive heart failure. Subsequent studies have confirmed beneficial effects of ACE inhibitors in reduction of morbidity and mortality rates and improvement in the quality of life in patients. Therefore, treatment based on ACE inhibition has become an established therapy for patients with chronic heart failure (CHF). The effects of ACE inhibitors on CHF are mostly attributed to the blockade of angiotensin II (Ang II) production, which induces cardiomyocyte hypertrophy after MI or heart failure. It has been shown that Ang II stimulates collagen synthesis in cultured cardiac fibroblasts and induces apoptosis in cardiomyocytes and endothelial cells. Collectively, these observations support a role of Ang II in promoting the progression of cardiac remodeling and suppressing cardiac function. Since ACE is the same enzyme as kininase II, a kinin-degrading enzyme, inhibition of ACE not only results in reduced Ang II levels but also decreased kinin breakdown. This results in the accumulation of kinin in plasma and tissues. Previous reports have shown that a kinin B2 receptor antagonist, icatibant, partially abolished the protective effect of ACE inhibition in cardiac remodeling, implicating a role of kinin in cardioprotection. Binding of intact kinin to the kinin B2 receptor activates second messengers such as nitric oxide (NO)/cGMP and prostacyclin/cAMP and triggers many biological effects. Therefore, ACE inhibition could also be attributed to kinin-mediated protective effects in cardiac hemodynamics and remodeling. However, the potential mechanisms of the effects of kinin in cardiac remodeling have not been established.

Apoptosis in cardiomyocytes is one of the major factors that contributes to the progression of heart failure after MI. Recently, we reported that kallikrein gene delivery results in increased cardiac kinins and cGMP levels and reduced apoptosis after acute myocardial ischemia and reperfusion, and the effect is abolished by icatibant. However, the role of KKS on cardiac remodeling and apoptosis in progression of CHF is not well elucidated. In the present study, we investigated the potential role and mechanisms of the KKS in cardiac remodeling and apoptosis after MI by a somatic gene transfer approach.

Methods

Preparation of Adenovirus Carrying the Human Tissue Kallikrein Gene

Adenovirus containing the human tissue kallikrein gene under the control of cytomegalovirus promoter (Ad.CMV-cHK) was generated...
as previously described. Large quantities of high-titer Ad.CMV-cHK and control virus containing the luciferase gene (Ad.CMV-Luc) were prepared and purified for in vivo gene delivery.

**Animals and Treatments**

Wistar rats (male, 225 to 250 g body weight, Sprague-Dawley Harlan, Indianapolis, Ind.) were subjected to ligation of the left coronary artery as previously described. 22 One week after coronary artery ligation, the rats were randomly divided into two groups and injected through the tail vein with $1.2 \times 10^{10}$ plaque-forming units of adenovirus harboring either human tissue kallikrein (MI-cHK group) or luciferase gene (MI-Luc group). The sham rats underwent the same surgical procedure without ligation of the left coronary artery. At 2 or 6 weeks after coronary artery ligation, the hemodynamic parameters were analyzed and the animals were then euthanized. Tissues were harvested for morphological and biochemical analyses.

**Hemodynamic Parameters**

At the end of the study (2 or 6 weeks after coronary artery ligation), rats were anesthetized with sodium pentobarbital (50 mg/kg IP) and cardiac function and regional blood flow were measured by a fluorescent microsphere assay. The right carotid artery (RCA) and the left femoral artery (LFA) were catheterized with PE-50 tubing. The distal end of the cannula of the RCA was connected to a physiological pressure transducer (Statham Laboratories) coupled with a model 7E polygraph (Grass Instrument Co). After measurements of heart rate (HR) and mean arterial pressure (MAP), the tip of the RCA catheter was gently advanced into the LV, and LV end-diastolic pressure (LVEDP) was recorded. Then, 0.2 mL of solution containing fluorescent microsphere beads (2.0 $\times$ 10$^6$ beads, Molecular Probes, Inc) was injected within 10 seconds into the LV followed by 0.5 mL of saline. The reference sample for blood flow measurements was taken from the LFA and collected in a glass syringe at a flow rate of 0.68 mL/min for 90 seconds, sampling beginning 10 seconds before the injection of microspheres. When hemodynamic analyses were completed, the animals were euthanized and the heart and kidneys were removed and weighed. The blood, kidney, and heart were processed by the sedimentation method according to the manufacturer’s instructions. Cardiac output (CO) was calculated as $CO = Q_r \times Fluo_{r}$, where $Q_r$ is reference blood flow, $Fluo_r$ is total injected fluorescence, and $Fluo_r$ is reference fluorescence. CO was correlated to body weight and expressed as cardiac index (CI) = $CO/\text{weight}$. Total peripheral resistance index (TPRI) was calculated as $\text{MAP} \times \text{CI}^{-1}$. Regional blood flow (mL/min per gram) to tissue weight was calculated as ($Fluo_{tissue}/Wt_{tissue}$)($Q_r/Fluo_r$)100, where $Fluo_{tissue}$ is fluorescence in a tissue and $Wt_{tissue}$ is tissue weight.

**Dobutamine Stress Test**

To measure cardiac responses to dobutamine-induced stress, another group of rats was anesthetized with sodium pentobarbital (50 mg/kg IP) at 6 weeks after MI. Basal level of cardiac function was measured by the microsphere assay described above. Dobutamine (Sigma, 10 $\mu$g/kg per minute) was infused from the left jugular vein for 5 minutes and then cardiac function was assessed again. Before and during infusion of dobutamine, LV pressure was monitored with the use of an MP100 system (Biopac Systems, Inc). The data were evaluated by percent increase of $dP/dt$ and CI as compared with basal levels (resting condition).

**Cardiac Morphological Parameters**

At 2 and 6 weeks after MI, body weight (BW), heart weight (HW) and left ventricular weight (LVW) were measured and expressed as HW/BW and LVW/BW values, and left ventricular long axis (LVL; the length from apex to aortic valve in the heart) was also measured. The left ventricle was cut into 4 transversal slices (1 basal, 2 mid and 1 apical, 2 mm thick each). After fixation with 4% paraformaldehyde, the tissues were dehydrated and embedded. Four-micron sections were obtained for morphological analyses. For measurements of infarct size and collagen density, the sections were stained with sirius red. Infarct size (%) was expressed as the ratio of the sum of external and internal perimeters of LV. The scar lengths and LV perimeters were directly determined from mean of two mid-ventricular slices. LV internal perimeter (LVIP) was also used as an indicator of LV dilatation. To evaluate the collagen density, 20 different fields in the endocardium of the viable LV wall were analyzed at $\times 200$ magnification. Collagen density (%) was expressed as the ratio of collagen area to myocardial area.

For the measurement of cardiomyocyte size, each slide stained with Gordon and Sweet silver staining was examined at $\times 400$ magnification. One hundred cardiomyocytes were randomly chosen and traced with the use of NIH image software. The area measured was averaged as the cardiomyocyte size.

The standard immunoperoxidase procedure with an avidin-biotin-peroxidase complex (Vectastain ABC kits, Vector Laboratory) was performed according to the manufacturer’s instructions. The sections were then developed in a solution containing 0.02% H$_2$O$_2$ and 0.1% diaminobenzidine tetrahydrochloride. Twenty different fields at $\times 400$ magnification were chosen, and the number of capillaries was counted. To measure apoptosis in the non-MI area, TUNEL staining (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay) was performed according to the manufacturer’s instructions (in situ cell death detection, POD, Roche). Twenty different fields at $\times 100$ magnification were chosen, and the number of TUNEL-positive cardiomyocytes was counted. The numbers were divided by cardiomyocyte index (total area divided by mean cardiomyocyte size) to obtain the ratio of apoptosis in cardiomyocytes of non-MI area.

**Western Blot Analysis and Nitrite/Nitrate Levels in Heart Tissues**

At 2 weeks after coronary artery ligation, rats were anesthetized and noninfarcted LV was dissected. The tissues were homogenized (Polytron, Brinkmann Instruments) in 500 $\mu$L of 25 mMol/L Tris-HCl (pH 7.4) containing 1% Triton X-100, 0.1% SDS, 2 mMol/L EDTA, and 1% protease inhibitor cocktail (Sigma) and centrifuged at 14 000 rpm for 30 minutes at 4°C. The supernatants were used for Western blot analysis, with specific antibodies used for phospho-Akt, total Akt, and cleaved caspase-3 (Cell Signaling Technology). Nitrite/nitrate levels in the tissue extracts were measured by a fluorometric assay as previously described, and protein concentrations were determined by Lowry’s method.

**Statistical Analysis**

Values are expressed as mean±SEM. Statistical comparisons were performed with the use of 1-way ANOVA followed by the Fisher’s PLSD test for multiple comparisons. Regression analysis was used to compare the relation between TUNEL-positive cardiomyocytes and cardiomyocyte size or LV internal perimeter. A value of $P<0.05$ was considered statistically significant.

**Results**

**Effects of Kallikrein Gene Delivery on Infarct Size and Physiological and Hemodynamic Parameters After MI**

Our results show that mean infarct size did not differ significantly between the groups injected with the kallikrein or luciferase gene (37.9±2.3% in MI-Luc versus 38.5±3.2% in MI-cHK at 2 weeks after MI, 42.3±2.5% in MI-Luc versus 43.1±2.9% in MI-cHK at 6 weeks after MI). The Table shows physiological and hemodynamic parameters in hearts at 2 and 6 weeks after MI. There were no differences in BW among the groups at 6 weeks after MI. However, BW in the MI-Luc and MI-cHK groups was reduced as compared with the sham group at 2 weeks after MI. MI resulted in increases in HW, HW/BW, LVW, and LVW/BW, especially at 6 weeks.
Although there was no significant difference in responses to Dobutamine-Induced Stress, Kallikrein Gene Transfer Improves Cardiac Function compared with the sham group gradually decreased from 2 weeks to 6 weeks. Although the reduction of CO in MI-Luc was significantly lower compared with the sham group at 2 weeks (249.6 ± 21.6 mL/min per kilogram, n = 5, P < 0.05), whereas there was no significant difference was observed between MI-cHK (328.5 ± 31.0 mL/min per kilogram, n = 6) and sham (Figure 1C). The cardiac response to dobutamine on CI in MI-Luc rats was reduced as compared with that of sham rats after MI. Kallikrein gene delivery tended to reduce LVW and LVW/BW at 6 weeks after MI. Kallikrein gene delivery significantly decreased these parameters. MAP in MI-Luc and MI-cHK groups was significantly lower than in the sham group (249.6 ± 21.6 versus 357.9 ± 21.6 mL/min per kilogram, n = 5, P < 0.05), whereas no significant difference was observed between MI-cHK (328.5 ± 31.0 mL/min per kilogram, n = 6) and sham (Figure 1C). The cardiac response to dobutamine in MI-Luc after dobutamine infusion was significantly lower than that of sham (249.6 ± 21.6 versus 357.9 ± 21.6 mL/min per kilogram, n = 5, P < 0.05), whereas no significant difference was observed between MI-cHK (328.5 ± 31.0 mL/min per kilogram, n = 6) and sham (Figure 1C). The cardiac response to dobutamine on CI in MI-Luc rats was reduced as compared with that of sham rats.

Kallikrein Gene Transfer Improves Cardiac Responses to Dobutamine-Induced Stress
Although there was no significant difference in dP/dt before dobutamine infusion among sham, MI-Luc, and MI-cHK groups, positive dP/dt in MI-Luc after dobutamine infusion was significantly lower than that of the sham group (8141 ± 1144 versus 12 270 ± 1306 mm Hg/s, n = 5, P < 0.05), whereas no significant difference was observed between MI-cHK (10 045 ± 858 mm Hg/s, n = 6) and sham groups (Figure 1A). The cardiac response to dobutamine in MI-Luc on positive dP/dt was reduced as compared with that of the sham group (+8.0 ± 5.4 versus +36.6 ± 6.5%, n = 5, P < 0.05), whereas kallikrein gene delivery improved the response to dobutamine-induced stress (+30.7 ± 11.2%, n = 6) (Figure 1B). Similarly, although there was no significant difference in CI before dobutamine infusion between sham, MI-Luc, and MI-cHK, CI in MI-Luc after dobutamine infusion was significantly lower than that of sham (249.6 ± 21.6 versus 357.9 ± 21.6 mL/min per kilogram, n = 5, P < 0.05), whereas no significant difference was observed between MI-cHK (328.5 ± 31.0 mL/min per kilogram, n = 6) and sham (Figure 1C). The cardiac response to dobutamine on CI in MI-Luc rats was reduced as compared with that of sham rats.
left ventricular blood flow in MI-Luc was significantly
decreased compared with sham rats (3.2 ± 0.5 versus 5.9 ± 1.0
mL/min/g, n = 6 and 5, P < 0.05), whereas kallikrein gene
delivery significantly increased blood flow (4.8 ± 0.4 mL/
min/g, n = 6) (Figure 4A). LV resistance increased in MI-Luc
compared with the sham control (39.1 ± 8.9 versus
5.9 ± 21.9 ± 3.7 mm Hg/min/g per milliliter, n = 6 and 5,
P < 0.05), and kallikrein gene delivery significantly decreased
this parameter (21.3 ± 2.7 mm Hg/min/g per milliliter, n = 6,
P < 0.05) (Figure 4B). Nitrite/nitrate levels in LV in MI-Luc
were significantly lower than sham (157 ± 10 versus 201 ± 10
pmol/mg of protein, n = 6 and 5, P < 0.05), and kallikrein gene
delivery increased nitrite/nitrate production in LV (198 ± 5
pmol/mg of protein, n = 6) (Figure 4C).

Apoptosis and Apoptosis-Related Protein
Expression in LV

TUNEL-positive myocytes in the MI-Luc group at 2 weeks
after MI were significantly increased compared with the sham group,
whereas kallikrein gene delivery significantly reduced TUNEL-
positive myocytes (n = 6, P < 0.05) (Figure 5A). There were
positive correlations between TUNEL-positive myocytes and
cardiomyocyte size (n = 16, R = 0.85, P < 0.01) or LV internal
perimeter (n = 16, R = 0.65, P < 0.01) (Figures 5B and 5C). MI
significantly reduced the phosphorylation of the survival factor
Akt, whereas kallikrein gene delivery increased phospho-Akt
but had no effect on total Akt (Figure 6A). MI resulted in
increased cleaved caspase-3, whereas kallikrein gene delivery
reduced caspase-3 activation (Figure 6B).

Discussion

In the present study, we showed that kallikrein gene delivery
improves cardiac function and protects against cardiac remodeling
in CHF. The beneficial effects of kallikrein gene
transfer in the heart after MI include (1) attenuation of cardiac
hypertrophy, fibrosis, and LV enlargement; (2) increase in
capillary density; (3) improvement of cardiac responses to
dobutamine infusion; (4) inhibition of myocardial apoptosis;
and (5) enhancement of endothelial function including in-
creased blood flow, decreased vascular resistance, and in-
creased NO production. These combined results indicate that
the KKS prevents progression of heart failure and improves endothelial function through reduction of cardiac hypertrophy, fibrosis, and apoptosis.

In this study, we used dobutamine, a β1-adrenergic agonist, to mimic exercise-induced stress and showed that hearts remodeled after MI exhibit a low response to dobutamine. Interestingly, kallikrein gene delivery improved cardiac responses against dobutamine infusion (Figure 1). These results indicate that hearts remodeled after MI have low cardiac reserve, and kallikrein gene delivery can improve cardiac reserve. Patients with heart failure must limit their exercises or activities because heart failure symptoms are induced by such stress conditions caused by low cardiac reserve. Therefore, cardiac responses against stress are more important than the basal level of cardiac function. Taken together, these findings indicate that KKS may play an important role in improving exercise capacity for patients with heart failure after MI.

Kallikrein gene transfer not only reduced cardiac hypertrophy and fibrosis but also increased capillary density after MI. Capillary density in the heart appeared to be especially important under stress conditions because the demand of oxygen and nutrition is increased in the heart during stress. A previous study by Liu et al16 showed that MI induced the reduction of capillary density in non-MI area, and an ACE inhibitor prevented this reduction; however, kinin B2 receptor antagonist abolished the protective effect of ACE inhibition. These combined results indicate that kinin may promote an increase in capillary density. Increased Ang II levels in heart failure after MI may induce endothelial cell apoptosis and thus reduce capillary density in the heart.13 Our previous results together with the present study show that kallikrein gene delivery significantly increases cardiac kinin and NO levels. NO has been shown to inhibit human endothelial cell apoptosis.26 Therefore, kallikrein gene delivery may increase cardiac capillary density through suppression of endothelial cell apoptosis mediated by the kinin-NO pathway. Moreover, we have also reported that kallikrein gene delivery promotes spontaneous angiogenesis in hindlimb ischemia of rats.27 Thus, increased capillary density in hearts remodeled hearts after kallikrein gene transfer may contribute to enhanced endothelial function and reduced cardiac remodeling and apoptosis.

Myocardial apoptosis has been shown to be one of many important factors leading to cardiac remodeling and heart failure in several experimental and clinical heart failure stages.17,18 In this study, we observed positive correlations between TUNEL-positive myocytes and LV internal perim-

Figure 4. Effect of kallikrein gene delivery on cardiac physiological and biochemical parameters in LV 2 weeks after MI. A, Blood flow (mL/min per gram); B, vascular resistance (mm Hg/min·g·milli-liter); C, nitrite/nitrate levels (pmol/mg protein). Results are expressed as mean±SEM (n=5 to 10).

Figure 5. Effect of kallikrein gene delivery on myocardial apoptosis. A, TUNEL-positive myocytes/10³; B, correlation between TUNEL-positive myocytes and cardiomyocyte size; C, correlation between TUNEL-positive myocytes and LV internal perimeter. Results are expressed as mean±SEM (n=4 to 5).
increase myocardial apoptosis in heart remodelled heart after repeated injections. This may be one of the mechanisms to account for the increased phosphorylation of Akt and NO production also decreased in absence of repeated injections.

Results observed on week 6 after surgery represent the sustained effects of a single kallikrein gene delivery. The results observed at week 2 after surgery represent the early effect of kallikrein gene delivery. The time course selected was 2 and 6 weeks after coronary artery ligation or 1 and 5 weeks after gene delivery. Thus the time course was designed to analyze the acute and chronic effects of Ad-kallikrein gene delivery. The results observed at week 2 after surgery represent the early effect of kallikrein gene delivery. The results observed on week 6 after surgery represent the long-term accumulated effects of kallikrein gene delivery. Therefore, the second set of data (week 6) emphasizes the sustained effects of a single kallikrein gene delivery in the absence of repeated injections.

We evaluated the expression level of recombinant human tissue kallikrein in rats at only one point (3 days after gene delivery) to check whether the virus injection was successful. The time course of recombinant human tissue kallikrein levels in serum was not monitored continuously. However, we have shown that recombinant human tissue kallikrein in rat serum remains detectable on day 36 after tail vein injection as compared with control animals injected with the vehicle.

Figure 6. Effect of kallikrein gene delivery on Akt and cleaved caspase-3 activation in noninfarcted LV at 2 weeks after MI. A, Western blot analysis of phospho-Akt and total Akt (top), quantitative analysis of phospho-Akt, and total Akt (% of sham) (Bottom); B, Western blot analysis of cleaved caspase-3 (top), quantitative analysis of cleaved caspase-3 (% of sham).

In conclusion, this study shows that KKS plays an important role in attenuating heart failure and improving cardiac reserves by the reduction of cardiac remodeling after MI through the enhancement of endothelial function and the inhibition of myocardial apoptosis through the Akt signaling pathway.

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References


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