Increased Expression of Integrin-Linked Kinase Attenuates Left Ventricular Remodeling and Improves Cardiac Function After Myocardial Infarction

Liang Ding, MD; Li Dong, MD; Xin Chen, MD; Lujie Zhang, MD; Xiaoyu Xu, MS; Albert Ferro, FRCP, PhD; Biao Xu, MD, PhD

Background.—Left ventricular (LV) remodeling is associated with the development of heart failure after myocardial infarction. Here we investigated whether integrin-linked kinase (ILK) may regulate LV remodeling and function after myocardial infarction.

Methods and Results.—Adenoviral vector expressing ILK (n = 25) or empty adeno-null (n = 25) was injected into rat peri-infarct myocardium after left anterior descending coronary artery ligation. ILK expression was confirmed by Western blotting and immunofluorescence. Echocardiographic and hemodynamic analyses demonstrated relatively preserved cardiac function in adeno-ILK animals. ILK treatment was associated with reduced infarct scar size, increased scar thinning ratio, and preserved LV diameter, wall thickness, cardiomyocyte size, and myofilament density. Enhanced angiogenesis and reduced fibrosis were observed in the adeno-ILK group, along with reduced apoptosis as demonstrated by terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling analysis. Moreover, increased cardiomyocyte proliferation was found in adeno-ILK animals, as measured by proliferating cell nuclear antigen, Ki-67, and phosphohistone-H3 staining. At long-term follow-up, most indices of cardiac function and hemodynamics showed no difference between adeno-ILK and control animals by 9 weeks, although LV end-systolic diameter and infarct scar size were reduced in the adeno-ILK group at this time point. Additionally, ILK overexpression was found to exert a rescue effect on remodeling when administered in a delayed fashion 1 week after coronary artery ligation.

Conclusions.—ILK gene therapy improves cardiac remodeling and function in rats after myocardial infarction and is associated with increased angiogenesis, reduced apoptosis, and increased cardiomyocyte proliferation. This may represent a new approach to the treatment of postinfarct remodeling and subsequent heart failure. (Circulation. 2009;120:764-773.)

Key Words: angiogenesis ■ apoptosis ■ gene therapy ■ myocardial infarction ■ remodeling

Heart failure is an increasing public health problem worldwide, with the most common cause currently being cardiac remodeling after myocardial infarction (MI).1,2 Cardiomyocyte death in the context of MI in combination with increased preload and afterload triggers a cascade of biochemical intracellular signaling processes that modulate cardiac remodeling (which includes dilatation, hypertrophy, and fibrosis),3 and prevention or attenuation of these signaling processes is an important therapeutic goal.

Clinical Perspective on p 773

Integrin-linked kinase (ILK), a widely expressed serine/threonine protein kinase, is highly expressed in the heart.4 Previous studies have shown that ILK plays an important role in transducing cell–matrix interaction–induced biomechanical signals via binding to the cytoplasmic domain of β-integrins, which regulate cytoskeletal remodeling and angiogenesis, as well as cell growth, proliferation, survival, and differentiation.4,5 Recently, ILK has been reported to be an important factor regulating cardiac contractility,6 compensatory hypertrophy,7 survival, and repair.8 Targeted ILK deletion in the murine heart causes spontaneous dilated cardiomyopathy and heart failure.9 Additionally, ILK controls recruitment of endothelial progenitor cells to ischemic tissue.10 However, whether ILK has therapeutic potential after MI is not clear.
We hypothesized that ILK gene therapy would attenuate remodeling and improve cardiac function after MI. The purpose of this study was therefore to investigate the effect of ILK overexpression in the peri-infarct area on markers of cardiac ischemic damage, angiogenesis, and function.

**Methods**

**Recombinant Adenovirus Construction**

Wild-type ILK-encoding plasmid was a gift from Dr Hyo-Soo Kim (Seoul National University College of Medicine). Recombinant adenoviral vector (E1, E3 deleted) harboring human wild-type ILK cDNA and humanized recombinant green fluorescent protein (hrGFP) driven by the cytomegalovirus promoter were made by the GenScript Corporation (China Branch, Nanjing, China). Corresponding virus with null content was used as a control.

**Animal Model of MI and Adenoviral Vector Delivery**

Animal experiments were performed following the guidelines in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (National Institutes of Health publication No. 85-23, revised 1985). Adult, male Sprague-Dawley rats (n=50) weighing 180 to 200 g were anesthetized by intraperitoneal administration of a mixture of ketamine hydrochloride (50 mg/kg) and diazeepam (5 mg/kg). Rats were endotracheally intubated and mechanically ventilated (Jiangxi Teli, China) with supplemental oxygen. The heart was exposed through a thoracotomy at the left fourth intercostal space, and the left anterior descending coronary artery (LAD) was ligated by a 6-0 silk suture 1 to 2 mm below the tip of the left atrial appendage. After ligation, lungs were fully inflated by positive end-expiratory pressure. During ligation of the LAD, rats were randomized to treatment with either adeno-ILK or adeno-null; immediately after LAD ligation, a total of 100 μL adeno-ILK (2×10⁹ viral particles) or adeno-null (2×10⁹ viral particles) was injected into 5 regions in the peri-infarct zone with the use of a syringe attached to a 29-gauge needle. The chest cavity, muscles, and skin were sutured in 3 layers.

**Protein Expression After Adenoviral Vector Delivery**

In vivo expression of exogenous protein after adenoviral delivery was detected by Western blotting and immunofluorescence analysis. hrGFP was used as a marker for exogenous gene expression. Full details are given in the expanded Methods section in the online-only Data Supplement.

**ILK Kinase Assay**

ILK kinase activity was determined by in vitro kinase assays as described in the online-only Data Supplement. The level of in vivo phosphorylated Akt1/PKBa (a downstream target of ILK) was further confirmed by Western blotting analysis.

**Echocardiography and Hemodynamic Assessment**

Four weeks after viral delivery, cardiac function was evaluated by transthoracic echocardiography. Thereafter, cardiac catheterization was performed in animals for hemodynamic study. All measurements were performed by an observer blinded to treatment. Full details are given in the expanded Methods section in the online-only Data Supplement.

**Tissue Section Preparation**

After hemodynamic evaluation, heart samples were collected and used for histological, immunohistochemical, and terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) examination, as fully described in the online-only Data Supplement.

**Histological Analysis**

Paraffin-embedded sections were stained with hematoxylin-eosin for morphological examination or Masson’s trichrome for assessment of interstitial fibrosis. Infarct scar size, thinning ratio, left ventricular (LV) thickness, cardiomyocyte size, myofilament density, collagen volume fraction, and LV diameter were measured as described in the online-only Data Supplement.

**Analysis of Microvessel Density**

Microvessel density in the border area was evaluated by CD31 staining performed on frozen sections. Full details on the analysis of microvessel density can be found in the expanded Methods section in the online-only Data Supplement.

**Cardiomyocyte Apoptosis**

Cardiomyocyte apoptosis was measured by triple immunofluorescence staining of TUNEL, α-sarcomeric actin, and DAPI (see the online-only Data Supplement).

**Immunohistochemical Analysis of Cardiomyocyte Proliferation**

To detect whether ILK promotes cardiomyocyte proliferation, immunohistochemical analysis was performed for phospho-histone H3 (Ser10), proliferating cell nuclear antigen, and Ki-67. Full details of these procedures are in the online-only Data Supplement.

**Longer-Term Follow-Up and Effect of Delayed ILK Administration**

To investigate whether the improvement seen with ILK overexpression after MI is sustained or temporary, a subset of animals (n=6 per group) was intramyocardially injected with adenovirus-containing plasmid and examined by echocardiography 7 weeks and 9 weeks after adenoviral delivery. Hemodynamics and histology were evaluated 9 weeks after delivery.

To ascertain whether ILK overexpression can also have a rescue effect on the remodeling process, a separate cohort of rats (n=6 per group) was injected with adenovirus-containing plasmid intramyocardially in the border zone 1 week after LAD ligation. Four weeks later, echocardiographic, hemodynamic, and histological analyses were performed.

**Statistical Analysis**

All values were expressed as mean±SEM. All data analysis was performed with the use of SPSS 13.0 or STATA statistical software. Statistical significance was defined as P<0.05 (2-tailed). The normality or otherwise of distribution of the continuous variables was assessed with the Shapiro-Wilk test. Measurements in border or remote areas were compared within the adenovirus or control groups with the use of paired Student t-test (when values were normally distributed) or Wilcoxon signed rank test (when distributions were not normal). Because some echocardiographic parameters were obtained 7 and 9 weeks after gene transfer, when repeated measures were made, the generalized estimating equations statistical method with an independent correlation structure was used to estimate differences between groups. When significant differences between the 2 groups (P<0.05) over time were found by the generalized estimating equations approach, a post hoc comparison between 2 groups at each time point was made by Student t test with Bonferroni correction. Changes from baseline in echocardiographic parameters before and after delayed adenoviral delivery (4-week value minus baseline value) were compared between 2 groups by use of an unpaired Student t test. Comparisons of other parameters between groups were performed by unpaired Student t test (when distributions were normal) or Mann-Whitney U test (when distributions were not normal).

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.
Results
Fifty rats underwent LAD ligation and were injected with adeno-ILK or adeno-null, of which 42 survived (20 in the adeno-ILK group and 22 in the adeno-null group). Fifteen rats per group were randomly selected to undergo echocardiography, of which 1 rat in the adeno-null group died before echocardiographic examination could be performed as a result of anesthetic complications. After echocardiography, 10 rats per group were randomly selected to undergo hemodynamic testing, of which 1 rat in the adeno-null group died as a result of internal bleeding during the catheterization procedure. Forty rats (20 per group) were euthanized at the terminal time point. Forty hearts were used for frozen section (10 hearts per group × 2 groups) and paraffin section (10 hearts per group × 2 groups).

In Vivo Adenoviral Expression
After injection of adenovirus containing ILK and hrGFP or corresponding null vector in normal hearts, Western blotting analysis of ILK and hrGFP indicated higher ILK levels as well as expression of hrGFP levels in the adeno-ILK group compared with adeno-null controls between 3 and 28 days. In the adeno-ILK group, ILK and hrGFP protein levels rose progressively between 3 and 14 days after adenoviral delivery, after which ILK and hrGFP levels gradually declined and tapered off by 35 days (Figure 1A), indicating transient expression of this protein after treatment. Elevated expression of ILK as well as expression of hrGFP was also seen in infarcted hearts 3 days after adeno-ILK injection within the border area (Figure 1B), as defined by Evan’s blue dye staining (Figure I in the online-only Data Supplement).

Although Western blot analysis demonstrated successful expression of exogenous ILK, the morphological distribution of exogenous ILK was unknown. Therefore, heart slices from MI animals 5 days after transfection were stained for hrGFP. hrGFP-positive cells appeared in the border and noninfarct area around the injection sites. D, GFP expression is seen in α-actin+ cells and α-actin− cells, suggesting that adeno-ILK has been transfected into both cardiomyocytes (arrowheads) and noncardiomyocytes (Dc, arrows). α-Actin indicates α-sarcomeric actin; GFP, (humanized recombinant) green fluorescent protein; and DAPI, 4,6-diamidino-2-phenylindole. E, In vitro kinase assay activity of ILK and in vivo expression of phosphorylated Akt (a downstream target of ILK), which revealed an increased kinase activity after adeno-ILK delivery compared with null control. Bars=25 μm.

ILK Kinase Activity
We measured the in vitro kinase activity of ILK in infarcted hearts 3 days after adeno-ILK or adeno-null treatment (Figure 1E). The in vitro kinase assay revealed an increased ILK kinase activity in the adeno-ILK group compared with the adeno-null group, as evidenced by an increase in vitro phosphorylation of Akt. Western blot analysis with the use of denatured myocardial tissue lysate similarly showed an up-regulation of phospho-Akt in the adeno-ILK group, thereby confirming the in vitro findings.

Cardiac Function and Morphology
Macroscopically, the hearts of adeno-null rats were more spherical relative to those of adeno-ILK and untreated rats (Figure 2A), reflecting attenuation of LV global remodeling after adeno-ILK treatment.

Four weeks after adenoviral injection, echocardiography was used to evaluate cardiac function. The systolic function
index percent fractional shortening demonstrated significant improvement in the adeno-ILK animals compared with adeno-null animals (Table). In parallel with this, LV end-diastolic diameter and LV end-systolic diameter were both lower in adeno-ILK animals, with no significant difference in interventricular septal thickness at diastole or LV posterior wall thickness. Hemodynamic analysis substantiated the beneficial effects of adeno-ILK on cardiac function. An improvement was seen in both LV systolic pressure and LV end-diastolic pressure in adeno-ILK animals compared with adeno-null animals, with an increase in the former and a decrease in the latter (Figure 2B and 2C). The adeno-ILK group also had a greater maximum +dP/dt and a lower maximum −dP/dt than the control group (Figure 2D). Heart rate was not different between the 2 groups (Figure 2E).

After removal of the heart, LV diameter was measured at the level of the papillary muscle, and other parameters of ventricular morphology were acquired from 3 blocks (base, midregion, and apex; 5 slides per block) of each heart and averaged (Figure 3A). Cardiomyocyte size and myofilament density were analyzed in border and remote zones (Figure 3B). Infarct scar size was reduced in adeno-ILK animals compared with adeno-null animals, as manifested by a decrease in LV diameter (Figure 3D). Myofilament density was decreased in the border zone of adeno-null rats compared with adeno-ILK rats (Figure 3E). The thinning ratio was markedly increased in adeno-ILK compared with adeno-null animals (Figure 3F). Peri-infarct (border) zone thickness was lower in adeno-null animals than in adeno-ILK animals and was decreased to remote zone values in adeno-null rats (Figure 3G). Cardiomyocyte size was larger in adeno-null rats than in adeno-ILK rats within the border zone and was not different between the 2 groups within the remote zone (data not shown); in both adeno-null and adeno-ILK rats, cardiomyocyte size was considerably increased in the border region (Figure 3H).

Microvessel Density and Fibrosis
Masson’s trichrome staining for interstitial fibrosis in the border zone is shown in Figure 4A and 4B in the case of an adeno-null and an adeno-ILK rat, respectively. The border zone was confirmed by hematoxylin-eosin staining on serial sections (Figure 4C and 4D). Microvessel density was determined in the border zone by CD31 staining (Figure 4E and 4F). Collagen volume fraction was reduced in the adeno-ILK compared with the adeno-null group (Figure 4G), and microvessel density was found to be increased in adeno-ILK compared with adeno-null animals (Figure 4H).

Table. Echocardiographic Parameters 4 Weeks After Adenoviral Delivery

<table>
<thead>
<tr>
<th>Group</th>
<th>LVEDD, mm</th>
<th>LVESD, mm</th>
<th>%FS</th>
<th>IVSd, mm</th>
<th>LVPW, mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adeno-null (n=15)</td>
<td>7.81±0.26</td>
<td>4.71±0.24</td>
<td>39.84±2.01</td>
<td>1.36±0.04</td>
<td>1.43±0.045</td>
</tr>
<tr>
<td>Adeno-ILK (n=14)</td>
<td>6.94±0.21*</td>
<td>3.82±0.21*</td>
<td>45.45±1.63*</td>
<td>1.47±0.05</td>
<td>1.48±0.03</td>
</tr>
</tbody>
</table>

LVEDD indicates LV end-diastolic diameter; LVESD, LV end-systolic diameter; %FS, percent fractional shortening; IVSd, interventricular septal thickness at diastole; and LVPW, LV posterior wall thickness.

*P<0.05 vs adeno-null.
Cardiomyocyte Apoptosis
Cardiomyocyte apoptosis was quantified by TUNEL assay. In the border zone, the percentage of TUNEL-positive cardiomyocytes was markedly reduced in the adeno-ILK relative to the adeno-null group (Figure 5A and 5B). Interestingly, although the percentage of TUNEL-positive cardiomyocytes was low in the remote region of adeno-null rats, it was reduced to still lower levels in adeno-ILK animals (Figure 5B), suggesting that injection of adenovirus containing ILK in the peri-infarct region causes a reduction in cardiomyocyte apoptosis even in areas remote from this.

Cardiomyocyte Proliferation
Phosphohistone-H3 was found to localize to α-sarcomeric actin–stained cells, suggesting that this represented cardiomyocyte proliferation; in the border zone, phosphohistone-H3 was seen to a much greater level in adeno-ILK rats than in adeno-null animals (Figure 6A, 6B, and 6G), although in the remote zone it was present at a low level in both groups (Figure 6G).

Ki-67 expression (Figure 6C and 6D) as well as proliferating cell nuclear antigen expression (Figure 6E and 6F) localized to cardiomyocytes, and both were markedly upregulated in adeno-ILK animals compared with adeno-null rats (Figure 6H and 6I) in the border zone; interestingly, although proliferating cell nuclear antigen and Ki-67 expressions were low in the remote zone of both groups and comparable to levels seen in the border zone of adeno-null rats, expression of both was increased to a small but significant degree in the remote zone of adeno-ILK rats (Figure 6H and 6I). In the case of phosphohistone-H3, a trend was seen toward an increase in the remote zone of adeno-ILK animals, but this did not reach significance (Figure 6G). Collectively, these data confirm that injection of adenovirus containing ILK in the peri-infarct region gives rise to a marked increase in cardiomyocyte proliferation within that region and, additionally, a smaller increase in cardiomyocyte proliferation in more remote areas.

Longer-Term Follow-Up and Effect of Delayed ILK Administration
At 7 weeks after LAD ligation and adenoviral delivery, echocardiography revealed improved cardiac function (percent fractional shortening; Table I in the online-only Data Supplement) in the adeno-ILK compared with the adeno-null.
group; at 9 weeks, cardiac function (percent fractional shortening; Table I in the online-only Data Supplement) and hemodynamics (Figure IIC in the online-only Data Supplement) showed no difference between adeno-ILK and adeno-null animals, despite relative preservation of LV end-systolic diameter in the adeno-ILK group (Table I in the online-only Data Supplement). Morphological parameters were measured in paraffin-embedded heart sections with hematoxylin-eosin staining at 9 weeks (Figure IIB and IID to IIG in the online-only Data Supplement). The adeno-ILK animals showed a reduced infarct scar size compared with adeno-null animals (Figure IID in the online-only Data Supplement). There were no significant differences in LV diameter (Figure IIE in the online-only Data Supplement) and thinning ratio (Figure IIG in the online-only Data Supplement) between the 2 groups, although a trend to slight improvement in these parameters over adeno-null animals appeared to be present. In the adeno-ILK group, the wall thickness of the border area was increased, whereas that of the remote area was diminished compared with adeno-null animals (Figure IIF in the online-only Data Supplement).

In other animals, adenoviral injection was performed 1 week after LAD ligation to assess the potential of this therapy to rescue cardiac function and remodeling after MI. Four weeks after adenoviral injection, improved cardiac function was seen in the adeno-ILK group, with an enhanced percent fractional shortening (Table II in the online-only Data Supplement) and a greater maximum +dP/dt (Figure IIIC in the online-only Data Supplement).
Bars

myocytes in both border and remote zones of hearts from

in border zone. B, Quantification of TUNEL-positive cardio-

cellular, and molecular changes, 2 and these manifest as

LV remodeling after MI involves functional, geometric,
thinning ratio, decreased cardiomyocyte hypertrophy, and

Data Supplement), or thinning ratio (Figure IIIF in the online-only Data Supplement) compared with adeno-null animals. Relative to adeno-null animals, preserved LV diam-

eter and increased border zone wall thickness were observed in

the adeno-ILK group, although there was no statistical
difference in infarct scar size (Figure IIID in the online-only

and increased myofilament density, as well as attenuated intersti-
tial fibrosis and myocyte apoptosis. Preserved scar size and

Postinfarction remodeling has been divided into an early

within 72 hours) and a late phase (beyond 72 hours). 4

although we detected ILK expression from 3 days after

infection in our study, it is known that recombinant adeno-

viruses can produce physiologically significant levels of

transgene as early as 2 to 4 hours after infection, 15

rendering it possible that the effect of adeno-ILK in this system

commenced earlier than 3 days and within the early phase of

remodeling. Our results demonstrate that expression of ILK

itself and hrGFP disappeared by 35 days (5 weeks), whereas

the effect of ILK treatment persisted for at least 7 weeks, as

judged by hemodynamic alterations as well as structural and

morphological changes in the heart. Besides the therapeutic
effects of ILK injection at the time of coronary ligation, a

rescue effect of ILK overexpression was also seen on the

remodeling process when injected 1 week after coronary

ligation.

ILK has been shown to be a critical component of the
cardiac stretch sensor and plays an important role in integrin-

mediated mechanosensing/mechanotransduction in the heart. 6

It links membrane-bound β-integrins and the actin cytoskel-
eton to central signaling pathways, which have the effect of

preserving cardiac structure and function, including cardio-

myocyte contractility, postinfarct cell migration, and cell

attachment. 6,8,9 The results presented here suggest that ILK
treatment may similarly give rise to an increase in signaling

that beneficially influences remodeling in a variety of ways.

Bendig et al 6 showed that ILK regulates cardiomyocyte

contractility, whereas mutation of ILK impairs cardiac con-

tractility and reduces atrial natriuretic peptide and vascular

endothelial growth factor expression. Therefore, ILK overex-

pression is likely to enhance cardiomyocyte contractility as

well as atrial natriuretic peptide and vascular endothelial

growth factor expression after infarct, and atrial natriuretic

peptide could in turn reduce LV remodeling. 16

Moreover, ILK has been reported to contribute to angiogenesis as well as cell growth, proliferation, survival, and differentiation in

noncardiological studies, 5 which could also contribute to the

therapeutic effect of ILK in postinfarct remodeling. Our

results bear out the cardioprotective, proangiogenic, prolifer-

ative, and antiapoptotic effects of ILK.

Apoptosis contributes to the progression of MI, and anti-
apoptotic treatment at an early stage reduces the infarct size. 17

Myocardial apoptosis has been found to be a major determi-
nant of unfavorable LV remodeling. 18

Furthermore, increased apoptosis in areas remote from the infarct contributes to late

LV remodeling after MI. 19

Previous studies have indicated that ILK may reduce noncardiomyocyte cell apoptosis. 20

In the present study, we have found that ILK reduces cardio-

myocyte apoptosis, and this is true not just in the border zone

(where the adenovirus was injected) but also more remotely.

Overall, our results suggest that overexpression of ILK may

attenuate remodeling by reducing MI size as well as progres-

sive infarct extension and fibrous replacement secondary to

apoptosis.
One important component of the remodeling process is neoangiogenesis. After MI, neoangiogenesis is normally unable to compensate for the decreased blood supply and to support the tissue growth required for contractile compensation and the greater demands of the hypertrophied but viable myocardium; this may contribute to the death of otherwise viable myocardium, leading to progressive infarct extension and fibrous replacement. In the present study, we found an increase in angiogenesis in adeno-ILK animals, and this may contribute importantly to the reduction in remodeling. Lee et al. demonstrated that ILK as a hypoxia-responsive molecule may control the recruitment of endothelial progenitor cells to ischemic tissue. Moreover, Friedrich et al. found that ILK is crucial for vascular development via integrin-matrix interactions and endothelial cell survival. Additionally, ILK can promote vascular endothelial growth factor expression. In our model, therefore, we would predict that overexpression of ILK after vector injection may enhance local vascular endothelial growth factor expression, protect endothelial cells, promote endothelial progenitor cell recruitment, and improve vascular development, resulting in enhanced angiogenesis.

In the border zone of MI, differentiated cardiomyocytes have been shown to reenter the cell cycle and recommence proliferation, as assessed by increased cardiomyocyte DNA synthesis. Sustained cardiomyocyte replacement may attenuate the cell loss after MI, resulting in improved ventricular remodeling. Our data show enhanced cardiomyocyte proliferation in the border zone after ILK treatment. Yamabi et al. have shown that overexpressed ILK can promote stem cell amplification. It has also been reported that overexpression of ILK can induce the expression of cyclin D2, and cyclin D2 can promote regenerative growth in injured hearts. Furthermore, ILK may reduce apoptosis of viable cardiomyocytes after infarction, thereby contributing to cardiomyocyte proliferation. However, the precise mechanisms of ILK-induced cardiomyocyte proliferation need to be evaluated in further studies.

Although the majority of transfected cells were cardiomyocytes, some other transgene-expressing cells that look morphologically like fibroblasts were also seen. Previous studies have shown that cardiac fibroblasts can migrate from the border zone to the infarct zone after MI and thereby contribute to the postinfarct healing process by proliferation, collagen synthesis, and transformation into myofibroblasts, resulting in a firm scar. It has been reported that overexpression of wild-type ILK can protect fibroblasts from apoptosis, whereas ILK-deficient fibroblasts exhibit impaired cell migration and reduced proliferation rates. It is therefore possible that ILK overexpression may promote cardiac fibroblast viability, migration, and proliferation, which might in turn contribute to reduced infarct scar expansion. Further studies are needed to determine the precise role of ILK-overexpressing cardiac fibroblasts during remodeling.

In the present study, we found an increased kinase activity of ILK in the infarcted hearts after adeno-ILK delivery, which
might contribute to its therapeutic effect. However, whether ILK itself or its kinase activity is essential to the effect remains unclear from the present study, and further investigation using vectors expressing a kinase-dead ILK is required.

In previous studies, evaluation of efficacy in adenovirus-mediated gene therapy has usually been performed within 4 to 6 weeks. In the present study, we performed a longer (up to 9 weeks) follow-up study to investigate the long-term efficacy of adeno-ILK treatment. The data demonstrate that the improvement in cardiac function after adeno-ILK transfection persists up to at least 7 weeks, and, although cardiac function and hemodynamics were not different between adeno-ILK and adeno-null animals at 9 weeks, nevertheless LV end-systolic diameter and infarct scar size were reduced in the adeno-ILK group at this time point. In future studies, it would be useful to examine the usefulness of alternative vectors that can express the target gene for much longer periods of time at a high level, such as recombinant adeno-associated virus.  

Considering the potential for detrimental angiogenesis and hypertrophy in response to prolonged ILK expression, we also examined both macroscopically and histologically the liver, kidneys, and spleen from ILK-treated animals 4 and 9 weeks after treatment, but no instances of neoplasia were observed. However, longer observation intervals with larger numbers of animals are required to exclude the possibility of neoplastic transformation. Cellular and humoral immune responses to adenovirus-mediated gene therapy should also be assessed in future studies. Nevertheless, our observations suggest that an ILK overexpression strategy may be of therapeutic benefit in preventing the evolution of post-MI LV remodeling and heart failure.

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Disclosures

None.

References


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**CLINICAL PERSPECTIVE**

Postinfarction ventricular remodeling, consisting of changes in cardiac geometry and function, is an important cause of heart failure after myocardial infarction. Current clinical approaches to preventing this include early recanalization of the infarct-related artery and pharmacological interventions (for example, angiotensin converting-enzyme inhibition, β-blockade), but despite such approaches, progressive remodeling still occurs with resultant ventricular dysfunction. Integrin-linked kinase (ILK), a multifunctional protein kinase, has been reported to play important roles in regulating angiogenesis, cell proliferation, and survival and has also been shown to be crucial in the maintenance of cardiac structure and function in mice with cardiac-specific ILK ablation. However, to date, its role, if any, in postinfarction ventricular remodeling has not been shown. The present study indicates that overexpression of ILK by adenoviral gene transfer after myocardial infarction in rats attenuates cardiac remodeling and improves cardiac function. This is accompanied by enhanced neovascularization, reduced myocyte apoptosis, decreased fibrosis, and elevated cardiomyocyte proliferation. ILK also exhibits a rescue effect on the remodeling process after delayed delivery (1 week after myocardial infarction). Our findings suggest that interventions including gene therapy designed to increase ILK expression may be a promising therapeutic approach to prevent postinfarction ventricular remodeling and consequent heart failure.
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SUPPLEMENTAL MATERIAL

Increased Expression of Integrin-Linked Kinase Attenuates Left Ventricular Remodeling and Improves Cardiac Function after Myocardial Infarction

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Supplemental Methods

Protein Expression Following Adenoviral Vector Delivery

Control non-infarcted animals (n=6/group) were used to confirm in vivo protein expression following adenoviral vector delivery by intramyocardial injection with adeno-ILK or adeno-null vector into the cardiac apex (3 spots/heart). Three days, one week, two weeks, three weeks, four weeks and five weeks following injection, animals were sacrificed by CO2 inhalation and myocardial tissues were harvested. Myocardium was subsequently homogenized with a Dounce tissue grinder in lysis buffer consisting of: 10mmol/l Tris/HCl (pH 7.2), 150 mmol/l NaCl, 0.1% SDS, 1% (v/v) Triton X-100, 1% sodium deoxycholate, 5 mmol/l EDTA, 1 mmol/l Na3VO4, 50 mmol/l NaF , 0.2mmol/l phenylmethylsulfonyl fluoride and protease inhibitor cocktail (complete EDTA-free, Roche). Protein content was determined by BCA protein assay kit (Pierce). Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore). Western blots were probed with mouse anti-human ILK antibody (1:3000, BD Transduction Laboratories), rabbit anti-GFP antibody (1:800, Beyotime Institute of Biotechnology) or mouse anti-GAPDH (1:3000, Kangchen Bio-tech). Peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (1:10,000, Santa Cruz Biotechnology) was used as secondary antibody, and bands were visualized by enhanced chemiluminescense (Amersham) or Chemiluminescence HRP substrate (Millipore) in conjunction with BioMax films (Kodak).

In view of the possibility of increased bleeding and leakage in infarcted heart during
intramyocardial injection, protein expression following adenoviral vector delivery was also examined in infarcted hearts. Western blotting (n=5/group) as well as immunofluorescence (n=3/group) were performed using hearts on day 3 or day 5 from hearts following LAD ligation (infarcted heart, border zone and 5 spots). For Western blot analysis of ILK and hrGFP expression in the border area, tissue from the border area was obtained as described. Briefly, rats on day 3 were injected with 2% Evans Blue dye (Sigma) via the femoral vein to identify non-perfused myocardium. The infarct area (with no Evans Blue staining) and border area (2 mm of myocardium around the infarct area) were separated (Supplemental Figure I). For immunofluorescence, hearts on day 5 were harvested, fixed in 4% paraformaldehyde for 2 hr at 4°C, and immersed in 30% sucrose overnight at 4°C. Fixed tissues were embedded in Tissue-Tek O.C.T. (Sakura Finetechanical, Tokyo) and snap-frozen with liquid nitrogen. Frozen tissues were sectioned (6μm), fixed in cold acetone, then probed with a mixture of rabbit anti-GFP (1:100; Beyotime Institute of Biotechnolgy) and mouse anti-rabbit sarcomeric actin (1:50, Abcam). Alexa Fluor 488 goat anti-rabbit secondary antibody (1:200, Molecular Probes) and Alexa Fluor 555 goat anti-mouse secondary antibody (1:250, Molecular Probes) were used subsequently for detection by fluorescence microscopy.

**ILK Kinase Assay**

In vitro ILK kinase activity assay was performed as described. Briefly, border zone myocardial tissue was harvested three days after intramyocardial injection of adeno-ILK or adeno-null vector into the border zone in the animals who had undergone LAD coronary artery ligation. Myocardial lysates were immunoprecipitated using anti-ILK antibody (BD Laboratories) and
protein A/G PLUS-Agarose (Santa Cruz Biotechnology), overnight at 4°C according to the manufacturer’s protocols. The immunoprecipitated ILK was reacted with Akt1/PKBα (Upstate Biotechnology) for 60 min at 30°C, in kinase reaction buffer (50 mmol/l HEPES, pH 7.4, 10 mmol/l MnCl₂, 10 mmol/l MgCl₂, 2 mmol/l NaF, 1 mmol/l Na₃VO₄, 200 μmol/l ATP). Phosphorylation of Akt1/PKBα (a downstream target of ILK) was analyzed by immunoblotting with rabbit anti-phospho-Akt (Ser473) (Cell Signaling Technology) and rabbit anti-Akt (Cell Signaling Technology) antibodies.

**Echocardiography and Hemodynamic Assessment**

Four weeks after viral delivery, transthoracic echocardiography was performed using a cardiovascular ultrasound system (Vivid 7, General Electric Co.) with a 10-MHz linear-array transducer. Animals were weighed and anesthetized as described above and placed on a warm blanket. The cardiac long-axis and short-axis views were obtained in the 2-dimensional mode, and M-mode tracings were recorded. LV end-systolic dimension (LVESD), LV end-diastolic dimension (LVEDD), interventricular septal thickness in diastole (IVSd) and LV posterior wall thickness (LVPW), analyzed in the M-mode tracings at the papillary muscle level, were measured from at least three consecutive cardiac cycles. Percent LV fractional shortening (%FS) was calculated as:

\[
\%FS = \frac{(LVEDD-LVESD)}{LVEDD} \times 100 \%
\]

Following echocardiography, hemodynamic assessment was performed using a polyethylene catheter (PE 50, Becton-Dickinson) which was introduced into the LV via the right carotid
artery and connected to a pressure transducer equipped with a polygraph (RM6240, Chengdu Instrument Factory). LV systolic pressure (LVSP), LV end-diastolic pressure (LVEDP), maximum positive and negative dP/dt (+dP/dt\text{max}, -dP/dt\text{max}) and heart rates (HR) were recorded continuously. All measurements were performed by an observer blinded to treatment.

**Tissue Section Preparation**

After hemodynamic evaluation, hearts were arrested in diastole by intravenous administration of 2mol/l KCl, rapidly excised and washed in ice-cold saline. The myocardium was sliced into three blocks each of 4-5mm thickness, then snap-frozen in liquid nitrogen and stored at -80°C, or fixed in 4% buffered neutral formalin at room temperature for 8-12 hours. The frozen blocks were embedded in OCT and cut into 6 μm sections for subsequent analysis. The formalin-fixed tissue was embedded in paraffin wax and cut into 5 μm sections before use. Three tissue blocks (base, mid-region, and apex) were used for histological, immunohistochemical and TUNEL examination, and mean values were obtained for each heart.

**Histological Analysis**

Paraffin-embedded sections (from basal, mid-region, and apical blocks, 5 slices/block) were stained with hematoxylin-eosin for morphologic examination or Masson's trichrome for assessment of interstitial fibrosis. Infarct scar size, thinning ratio, LV thickness, cardiomyocyte size, myofilament density and collagen volume fraction (CVF) were measured and averaged across the three blocks for each animal, while LV diameter was measured at the level of the papillary muscle as described. Infarct scar size was expressed as the sum of the epicardial and
endocardial scar length divided by the sum of the LV epicardial and endocardial circumferences. Thinning ratio was determined as the ratio of mean thickness of infarcted wall to mean thickness of the normal wall. Myofilament density in the peri-infarct (border) zone was defined as the total number of myocytes per 20× high-power field. Cardiomyocyte size was analyzed as previously described. Thirty-five cardiomyocytes per slide were measured and averaged for each section. To determine whether ILK overexpression influences collagen fiber accumulation after infarction, we randomly selected 5 fields per slice and calculated CVF as the ratio of Masson's trichrome-stained collagen area to total myocardium area, as described. The morphologic parameters and CVF were measured using Image Pro Plus (Media Cybernetics), by a blinded observer.

Analysis of Microvessel Density
To measure microvessel density in the border area, frozen sections were stained with anti-mouse CD31 antibody (1:200, BD Pharmingen) and visualized by EnVision detection system with peroxidase/DAB (DAKO). Serial sections of frozen heart tissues were cut and stained with hematoxylin-eosin for morphologic identification of border and infarct area, and with CD31 staining for measuring microvessel density. Microvessel density was calculated as reported by Weidner et al. Three sections of each heart were measured and averaged.

TUNEL Assay
To detect apoptosis, TUNEL assay was performed on paraffin-embedded sections using DeadEnd Fluorimetric TUNEL System (Promega), according to the manufacturer’s directions.
Following this, sections were incubated with mouse monoclonal α-sarcomeric actin antibody (1:50, Abcam), which is specific for cardiac and skeletal muscle actin and does not detect smooth muscle actin. Alexa Fluor 555 goat anti-mouse antibody (1:250, Molecular Probes) was applied to detect the α-sarcomeric actin antibody. Cell nuclei were counterstained with DAPI (Sigma). The number of TUNEL-positive myocyte nuclei in the border and remote areas was manually counted. The total number of nuclei was determined by automated counting of the DAPI positive signals using Image Pro Plus software (Media Cybernetics).

**Immunohistochemical Analysis of Cardiomyocyte Proliferation**

To detect whether ILK promotes cardiomyocyte proliferation, immunohistochemical analysis was performed for phospho-histone H3 (1:100 rabbit anti-phospho-histone H3; Cell Signaling Technology), Proliferating Cell Nuclear Antigen (PCNA; 1:100 mouse anti-PCNA, Zymed Laboratories) and Ki-67 (1:100 mouse anti-Ki-67, Zymed Laboratories). After fixing in ice-cold acetone and washing in PBS, frozen sections were blocked with 5% normal goat serum and incubated with primary antibody mixture against phospho-histone H3 and α-sarcomeric actin (1:50, Abcam) overnight at 4°C, followed by secondary antibody mixture containing fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody (1:10, Kirkegaard & Perry Laboratories) and Alexa Fluor 555 goat anti-mouse antibody (1:250, Molecular Probes) for 1h at room temperature. Nuclei were counterstained with DAPI (Sigma). Dual-positive cardiomyocyte nuclei and total nuclei were quantitated in border and remote fields (×400 magnification, Imager A1, Zeiss, Germany). The ratio of phospho-histone H3-positive nuclei to the total number of nuclei was calculated and was averaged over at least ten fields per specimen.
Staining for PCNA and Ki-67 was performed on paraffin-embedded sections, then detected and visualized by EnVision detection system (DAKO). Only nuclei that were clearly located in cardiac myocytes were counted (×400 magnification, Zeiss Axiostar Plus).
**Supplemental Table I.** Echocardiographic parameters after adenoviral delivery.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>7 weeks</th>
<th>9 weeks</th>
<th>Coefficients # (95% confidence interval)</th>
<th>P #</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Group</td>
<td>Time</td>
<td>Group</td>
<td>Time</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>Adeno-null</td>
<td>7.50 ± 0.07</td>
<td>7.76 ± 0.12</td>
<td>-0.41</td>
<td>0.07</td>
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<tr>
<td></td>
<td>Adeno-ILK</td>
<td>7.20 ± 0.11*</td>
<td>7.24 ± 0.19</td>
<td>(-0.64 -0.18)</td>
<td>(-0.04 0.19)</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>Adeno-null</td>
<td>5.10 ± 0.10</td>
<td>5.34 ± 0.10</td>
<td>-0.49</td>
<td>0.12</td>
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<tr>
<td></td>
<td>Adeno-ILK</td>
<td>4.62 ± 0.07**</td>
<td>4.84 ± 0.10**</td>
<td>(-0.66 -0.32)</td>
<td>(0.03 0.20)</td>
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<tr>
<td>%FS</td>
<td>Adeno-null</td>
<td>32.11 ± 0.67</td>
<td>31.19 ± 0.65</td>
<td>2.82</td>
<td>-0.90</td>
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<tr>
<td></td>
<td>Adeno-ILK</td>
<td>35.82 ± 1.35*</td>
<td>33.13 ± 0.75</td>
<td>(1.17 4.48)</td>
<td>(-1.73 -0.08)</td>
</tr>
<tr>
<td>IVSd (mm)</td>
<td>Adeno-null</td>
<td>1.20 ± 0.03</td>
<td>1.23 ± 0.02</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Adeno-ILK</td>
<td>1.23 ± 0.02</td>
<td>1.25 ± 0.05</td>
<td>(-0.04 -0.07)</td>
<td>(-0.02 0.04)</td>
</tr>
<tr>
<td>LVPW (mm)</td>
<td>Adeno-null</td>
<td>1.21 ± 0.04</td>
<td>1.21 ± 0.02</td>
<td>-0.04</td>
<td>0.02</td>
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<tr>
<td></td>
<td>Adeno-ILK</td>
<td>1.22 ± 0.01</td>
<td>1.23 ± 0.04</td>
<td>(-0.09 -0.02)</td>
<td>(-0.01 0.05)</td>
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</tbody>
</table>

Animals (n=6/group) were transmyocardially injected with adeno-null or adeno-ILK immediately after LAD ligation. Echocardiographic parameters were obtained 7 weeks and 9 weeks after adenoviral delivery. In view of the repeated measures of outcome at different time points, the generalized estimating equations (GEE) statistical method was used to estimate differences between groups.

*P<0.05, **P<0.01 vs. adeno-null.  # evaluated by generalized estimating equations.
**Supplemental Table II.** Echocardiographic parameters before and after delayed adenoviral delivery.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>1 week post-infarct (before delivery, baseline)</th>
<th>4 weeks after delivery (change from baseline)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Adeno-null</td>
<td>Value</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>6.76 ± 0.46</td>
<td>6.61 ± 0.25</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>4.32 ± 0.38</td>
<td>4.16 ± 0.24</td>
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<tr>
<td>%FS</td>
<td>36.41 ± 1.79</td>
<td>37.25 ± 1.56</td>
</tr>
<tr>
<td>IVSd (mm)</td>
<td>1.2 ± 0.03</td>
<td>1.25 ± 0.04</td>
</tr>
<tr>
<td>LVPW (mm)</td>
<td>1.18 ± 0.02</td>
<td>1.20 ± 0.01</td>
</tr>
</tbody>
</table>

One week after LAD ligation, animals (n=6/group) underwent echocardiography. Thereafter, animals were injected with adeno-null or adeno-ILK in the border zone, and echocardiography was repeated 4 weeks after adenoviral delivery. At baseline, no significant differences were found between the two groups in LVEDD, LVESD, %FS, IVSd and LVPW.

*P<0.05 vs. adeno-null (change from baseline)..
Supplement Figure III

A. Adeno-null vs Adeno-ILK

B. Histological images of heart sections

C. Graph showing dp/dtmax (mmHg/s) with Adeno-null and Adeno-ILK

D. Bar graph showing Infarct Scar Size (%)

E. Bar graph showing LV Diameter (mm)

F. Bar graph showing LV Wall Thickness (mm) for Border and Remote areas

G. Bar graph showing Thinning Ratio (%)

Legend:
- Adeno-null
- Adeno-ILK
Supplemental Figure Legends

Supplemental Figure I. The border-zone tissues were identified and separated from infarcted heart using Evan's Blue dye. The non-Evan's Blue-staining area represents the infarcted area, while Evan's Blue-staining area represents non-infarcted heart. The border zone was defined as 2mm of myocardium around the infarct area.

Supplement Figure II. The therapeutic effect of adeno-ILK was not seen 9 weeks after MI. A, Hearts from adeno-null rats (top row) and adeno-ILK rats (bottom row). B, Paraffin-embedded sections (base, mid-region, and apex sequentially) of myocardium were stained with hematoxylin-eosin 9 weeks after adeno-null (top panels) or adeno-ILK (lower panels) delivery. C, Hemodynamic analysis (±dP/dtmax) 9 weeks after adenoviral delivery. D-G, Analysis of cardiac remodeling at 9 weeks by quantification of infarct scar size (D), LV diameter (E), LV wall thickness (F) and thinning ratio (G) in hematoxylin-eosin-stained sections. *P<0.05 versus adeno-null group. Scale bars: 5mm.

Supplement Figure III. Delayed delivery of adeno-ILK (1 week post-LAD ligation) results in an improvement in cardiac function and morphology 4 weeks later. A, Hearts from adeno-null rats (top row) and adeno-ILK rats (bottom row). B, Paraffin-embedded sections (base, mid-region, and apex sequentially) of myocardium were stained with hematoxylin-eosin at 4 weeks following transfection with adeno-null (top panels) or adeno-ILK (lower panels). C, Hemodynamic analysis (±dP/dtmax) at 4 weeks following adenoviral delivery. D-G, Evaluation
of cardiac remodeling at 4 weeks following adenoviral delivery, by quantification of infarct scar size (D), LV diameter (E), LV wall thickness (F) and thinning ratio (G) in hematoxylin-eosin-stained sections. *P<0.05 versus adeno-null group. Scale bars: 5mm.
Supplemental References


