Midkine gene transfer after myocardial infarction in rats prevents remodelling and ameliorates cardiac dysfunction

Arihiro Sumida††, Mitsuru Horiba†*†, Hisaaki Ishiguro†, Hiroharu Takenaka†, Norihiro Ueda†, Hiroaki Ooboshi4, Tobias Opthof5,6, Kenji Kadomatsu7, and Itsuo Kodama1

1Department of Cardiovascular Research, Research Institute of Environmental Medicine, Nagoya University, Huro-cho, Chikusa-ku, Nagoya 464-8601, Japan; 2Department of Cardiology, Nagoya University Graduate School of Medicine, Nagoya, Japan; 3Department of Cardiothoracic Surgery, Nagoya University Graduate School of Medicine, Nagoya, Japan; 4Department of Medicine and Clinical Science, Graduate School of Medical Sciences, Kyusyu University, Fukuoka, Japan; 5Experimental Cardiology Group, Center for Heart Failure Research, Academic Medical Center, Amsterdam, the Netherlands; 6Department of Medical Physiology, University Medical Center Utrecht, Utrecht, the Netherlands; and 7Department of Biochemistry, Nagoya University Graduate School of Medicine, Nagoya, Japan

Keywords
Gene transfer • Coronary disease • Infarction • Haemodynamics • Remodelling

Aim
We have previously reported that therapy with midkine (MK) has a protective effect in mouse models of myocardial infarction (MI) and ischemia/reperfusion. The underlying mechanism was proved to be anti-apoptosis and prevention of left ventricular (LV) remodelling following angiogenesis. Here we investigated the effects of overexpression of MK by adenoviral gene transfer on cardiac function and remodelling in an experimental rat MI model.

Methods and results
MI was created in male Wistar rats. Adenoviral vectors encoding mouse MK (AdMK) or β-galactosidase (AdLacZ; as controls) were injected in myocardium at the onset of MI. One week after injection, in vivo adenoviral gene expression was assessed by western blot and histological analysis. After echocardiographic analysis at 4 weeks and haemodynamic analysis at 6 weeks after MI, AdMK animals had better cardiac function compared with AdLacZ animals. Heart weight (HW) and relative HW of AdMK animals were not different from sham-operated animals after 6 weeks, pointing to a very potent effect in the prevention of ischemic cardiomyopathy. In histological studies at 6 weeks after MI, AdMK animals had less fibrosis in the non-infarcted myocardium and higher vascular density in the border-zone area compared with AdLacZ animals. AdMK animals had strongly upregulated levels of phosphorylated extracellular signal-regulated kinase, Akt, PI 3-kinase, and Bcl-2, whereas the level of Bax was downregulated compared with AdLacZ animals.

Conclusion
Overexpression of MK prevents LV remodelling and ameliorates LV dysfunction by anti-apoptotic and pro-angiogenic effects. MK gene transfer may provide a new therapeutic modality in ischemic cardiomyopathy and ischemic heart failure.

1. Introduction
Midkine (MK) is a heparin-binding growth factor involved in neural survival, carcinogenesis, and tissue repair. The mobilization of inflammatory cells is closely related to the angiogenic efficacy of MK. During embryogenesis, MK is highly expressed in the mid-gestational period and then decreases to birth. MK is expressed at the interface between mesenchyme and epithelium, and the expression is limited to adult organs where this interaction occurs/remains. Kidney and uterus are typical organs formed at the epithelial–mesenchymal interaction. In a normal heart, the expression of MK is very weak, thus MK is neither considered to be involved in normal cardiac development.
nor does it affect normal cardiac function. Myocardial infarction (MI) strongly induces MK expression in the area adjacent to the infarction within 6 h after myocardial ischemia. Preceding studies showed that MK reduces apoptosis via extracellular signal-regulated kinase (ERK) phosphorylation and by Bcl-2 activation in mice subjected to ischemia/reperfusion. In addition, we have recently reported that exogenous MK attenuates left ventricular (LV) remodelling and improves long-term survival after MI by enhancing angiogenesis and anti-apoptosis via the Akt/PI 3-kinase pathway. Intramyocardial injection of collagen gel including MK also attenuates cardiac remodelling after MI by its angiogenetic action. Adenoviral gene transfer has been applied in the treatment of ischemic myocardium in various experimental models. Adenoviral gene transfer of MK had protective effects in a rat middle cerebral artery occlusion model and decreased brain infarct volume. To date, it is unknown whether adenoviral vector transfer of MK has cardioprotective effects in myocardial ischemia. In the present study, we show that MK gene delivery is indeed effective in a rat MI model. In addition, we have elucidated the underlying molecular biological mechanism.

2. Methods

2.1 Adenoviral vector construction

Replication-deficient (E1,E3 deleted) recombinant adenoviral vectors containing mouse MK transgene (AdMK) and β-galactosidase transgene (AdLacZ) as control vectors, driven by the cytomegalovirus, were used in the present study. These vectors were constructed at the University of Iowa Gene Transfer Vector Core (Beverly L. Davidson) and obtained via Kyusyu University (Tetsuro Ago).

2.2 Animal model

Male Wistar rats (8–10 weeks old, weight 222–325 g) were obtained from Chubu Kagaku Shizai. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996), and were approved by the Animal Care and Use Committee of Nagoya University. The rats were anesthetized with intraperitoneal injection of ketamine 50 mg/kg and xylazine 10 mg/kg. A single investigator blinded to the treatment groups performed all measurements.

Full details of these procedures are given in the Supplementary material online.

2.4 Cardiac haemodynamics

Cardiac haemodynamics were measured with a 1.4 F micro-tipped catheter (Millar instruments). The rats were anaesthetized as for echocardiography (see above). Full details of these procedures are given in the Supplementary material online. Thereafter, the rats were euthanized by injection of 1 mL of KCl (1 mEq/mL) into the LV cavity to arrest the heart in diastole. The heart was harvested and stored for histological, morphological, and molecular biological analyses.

2.5 X-gal staining

Hearts were fixed in 4% paraformaldehyde in PBS for 2 h, 20% sucrose in PBS at 4°C overnight, 30% sucrose in PBS for 2 h, and sliced and embedded in optimal cutting temperature compound to freeze with liquid nitrogen. For X-gal staining, frozen sections (8-μm thick) perpendicular to the apex-base axis of the LV were made with a cryostat and stained with X-gal solution as previously described. The sections were rinsed in PBS and counterstained with neutral red before mounting. Full details of these procedures are given in the Supplementary material online.

2.6 Picrosirius red staining

After fixation with 4% paraformaldehyde in PBS, the hearts were sliced, embedded in paraffin and cut transversely into 6-μm thick sections. In addition to regular haematoxylin and eosin staining, the sections were stained with picrosirius red to analyse types I and III collagen accumulation as previously described. Collagen deposition in the infarcted and non-infarcted areas was calculated as the percentage of stained tissue in the summed area of muscular and connective tissue by densitometry using Scion Image 4.0.3 (Scion Corporation). Full details of these procedures are given in the Supplementary material online.

2.7 Immunohistochemistry

Paraffin sections (thickness 6 μm) were processed for the von Willebrand factor (vWF) immunohistochemical staining after having been deparaffinized. Antigen was retrieved as previously described. To detect microvascular density in the peri-infarct area, a rabbit anti-v-WF antibody was used as a primary antibody. Secondary antibodies were biotinylated using goat anti-rabbit IgG and a streptavidin-horseradish peroxidase (Blood Vessel Staining Kit, CHEMICON). Subsequently, the relevant antigen was visualized using an avidin–biotin peroxidase complex and 3,3′-diaminobenzidine tetrahydrochloride. Nuclei were stained with haematoxylin. The number of capillary vessels in the border-zone area was counted using light microscopy (Microphot-FXA, Nikon) by a blinded investigator in 10 randomly selected areas and subsequently averaged.

2.8 Western blotting analysis

Levels of MK, ERK-1/2, Akt, PI 3-kinase, Bcl-2, and Bax proteins in the border-zone of LV myocardium were assessed by western blot analysis. In total, LV myocardial homogenates were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis on a 10–12.5% polyacrylamide gel and proteins were electroblotted on the membranes (Atto). After blocking for 1 h, the membranes were incubated with primary and secondary antibodies in PBS-T or TBS-T solutions for 1 h each. Antibodies against MK, phospho-Akt/PKBα (Upstate), Akt/PKBα (Upstate), diphospho-ERK-1/2 (Sigma), ERK1/2 (Sigma), anti-PI3K (Upstate), Bax (Santa Cruz), and Bcl-2 (Santa Cruz)
were used as primary antibodies, respectively. Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Sigma) and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Jackson Immunoresearch) were used as secondary antibodies. Dynabeads Protein G (Dynal Biotech) was used for immunoprecipitation (IP). ECL Plus Detection Reagents (GE Healthcare) were used as enhancers and for band detection of each protein, CoolSaver 1.0 (Atto) was used. The intensity of bands was quantified by densitometry with CS analyser 3.0 (Atto).

2.9 Statistical analysis
Data analyses were performed with SPSS for Windows (version 16.0). All data were described as mean ± SEM. Comparisons between two groups were analysed using Student’s t-test. Statistical comparisons among the groups were performed by ANOVA with Bonferroni post-hoc tests. A value of $P < 0.05$ was considered as statistically significant.

3. Results
MI was made in 30 rats. One rat had died before adenoviral injection. Of the 29 rats, 15 rats were injected with AdLacZ and 14 with AdMK. Three of the 15 AdLacZ rats had died before echocardiography at 4 weeks after MI. Two of 14 AdMK rats had died before echocardiography. Therefore, both the AdLacZ MI group and the AdMK MI group consisted of 12 animals. All five rats which had died before analyses had a large amount of pleural effusion due to heart failure. Sham-operated 8 rats (without MI; sham) all survived. Thus, there were three groups of rats. Group 1 consisted of these eight sham-operated rats that were either injected with AdLacZ (sham + AdLacZ, \( n = 4 \)) or AdMK (sham + AdMK, \( n = 4 \)). Group 2 comprised rats that were injected with AdLacZ 30 min after occlusion of the LAD (the AdLacZ group, \( n = 12 \)) and group 3 consisted of rats injected with AdMK 30 min after occlusion (the AdMK group, \( n = 12 \)).

3.1 Adenoviral gene expression after intramyocardial injection
To confirm adenoviral infection and gene expression, control rats were sham operated. AdLacZ or AdMK rats were injected into the LV myocardium around the LAD. One week after injection, the rats were sacrificed to estimate adenoviral gene transfer. Cells were stained blue after X-gal staining at the injected sites of the LV in the AdLacZ group, indicating expression of the β-galactosidase gene in the injected LV myocardium. Scale bar represents 100 μm. (A) Representative X-gal staining with counterstaining neutral red for band detection of each protein, CoolSaver 1.0 (Atto). They were represented with relative intensity to 1 week.

3.2 Prevention of LV remodelling and amelioration of dysfunction after MI
There were no pre-operative differences among groups 1–3 before MI with left ventricular ejection fraction (LVEF) of 78.9 ± 7.3%. Echocardiography was repeated at 4 weeks after MI. There were no differences in any parameters between the two sham subgroups (sham + AdLacZ group and sham + AdMK group; Table 1). At 4 weeks after MI, the AdMK treated rats showed a smaller LV cavity and better contractile function compared with the AdLacZ rats (Figure 2A and B). LVEF and fractional shortening were significantly higher in the AdMK group compared with those in the AdLacZ group (Table 1). In addition, left ventricular end-diastolic dimension (LVEDD) and left ventricular end-systolic dimension (LVESD) were significantly smaller compared with the AdLacZ group. The border-zone wall
thickness (BWT) was larger in the AdMK group compared with the AdLacZ group. All parameters in the AdMK group were in-between those in the sham and AdLacZ groups (Table 1). Also, all differences among the three groups were significant except heart rate (Table 1). AdMK significantly mitigates all pathophysiological contractile changes caused by MI.

### 3.3 Haemodynamics and morphological analysis

Haemodynamic data were obtained at 6 weeks after MI by LV catheterization to assess the LV function and the degree of heart failure. There were no differences in any parameters between the two sham subgroups (sham + AdLacZ group and sham + AdMK group; Table 2). The AdMK group had higher maximum dP/dt (dP/dt_{max}) and lower minimum dP/dt (−dP/dt_{min}) compared with the AdLacZ group (Table 2). The AdMK group also had lower LVEDP compared with the AdLacZ group. The dP/dt_{max} − dP/dt_{min} and LVEDP values in the AdMK group were in-between those in the sham and AdLacZ groups (Table 2). Also, all differences between the three groups were significant (Table 2). In fact, from the almost three-fold increase in LVEDP caused by MI (from 2.1 mmHg in sham to 8.3 mmHg in AdLacZ group), only 16% remained in the AdMK group (Table 2). After AdMK injection, heart weight (HW) and heart weight/body weight (HW/BW) ratio were significantly lower than in the AdLacZ group, whereas the difference with the sham-operated group was insignificant, underscoring the high efficacy of MK against the development of ischemic cardiomyopathy.

### 3.4 Reduction of collagen deposition after MI

Picrosirius red staining was performed at 6 weeks after MI to visualize types I and III collagen deposition in LV cross-sections. Figure 3A shows collagen deposition in red and normal myocardium in yellow in a representative AdLacZ heart with MI. Figure 3B shows prominent reduction of collagen deposition in the non-infarcted myocardium in a heart from the AdMK group compared with the AdLacZ group (Figure 3A). Also, the infarct itself was much smaller (compare Figure 3B with A). At the border-zone area of the infarct LV myocardium, the AdMK group had a higher density of collagen deposition and a thicker fibrotic wall (Figure 3D) compared with the AdLacZ group (Figure 3C). In contrast, the AdMK group had less fibrosis in the non-infarct area (Figure 3F) compared with the AdLacZ group (Figure 3E). Figure 3G shows a quantification of areas of collagen deposition in the non-infarct areas in the hearts from the AdLacZ and AdMK groups. In the AdMK group, there was a reduction of more than 50% of collagen deposition compared with the AdLacZ group.

### 3.5 Enhancement of neovascularization

To quantify neovascularization in the peri-infarct area of the rat heart, vWF immunohistochemical staining was carried out at 6 weeks after MI. Figure 4A shows vWF positive staining (dark brown) in capillary vessels (red arrows) in the peri-infarct area of representative samples from the AdLacZ, AdMK, and control group. Control sections refer to sections from AdMK hearts stained without the primary antibody. Negative staining in these controls indicate that the immunostaining was specific (Figure 4A). The AdMK group had a 50% higher vWF positive capillary density in the peri-infarct area compared with the AdLacZ group (Figure 4B). Thus, AdMK enhances angiogenesis in the peri-infarct area after MI.

#### Table 1 Echocardiographic data at 4 weeks after MI

<table>
<thead>
<tr>
<th></th>
<th>Sham-operated rats (n = 8)</th>
<th>AdLacZ rats (n = 12)</th>
<th>AdMK rats (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR, b.p.m</td>
<td>276 ± 17</td>
<td>280 ± 22</td>
<td>278 ± 19</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>73.5 ± 4.4</td>
<td>75.0 ± 3.7</td>
<td>74.3 ± 3.9</td>
</tr>
<tr>
<td>FS, %</td>
<td>37.5 ± 3.7</td>
<td>39.3 ± 3.4</td>
<td>38.4 ± 3.4</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>6.9 ± 0.4</td>
<td>7.1 ± 0.8</td>
<td>7.0 ± 0.6</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>4.2 ± 0.3</td>
<td>4.2 ± 0.5</td>
<td>4.2 ± 0.4</td>
</tr>
<tr>
<td>BWT, mm</td>
<td>1.3 ± 0.4</td>
<td>1.4 ± 0.1</td>
<td>1.3 ± 0.3</td>
</tr>
</tbody>
</table>

Sham + AdLacZ means sham-operated rats injected with AdLacZ. Sham + AdMK means sham-operated rats injected with AdMK. LVEF, left ventricular ejection fraction; FS, fractional shortening; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; BWT, border-zone wall thickness. LVEF = (LVEDD² − LVESD²)/LVEDD × 100 and FS = (LVEDD − LVESD)/LVEDD. Values are mean ± SEM.

*P < 0.001 vs. sham-operated rats.
**P < 0.001 vs. AdLacZ rats.
3.6 Upregulation of PI3k/Akt and ERK pathways

One week after MI, border-zone myocardium was collected for western blot analysis. To elucidate the mechanism of angiogenesis, phosphorylation of Akt and ERK-1/2 and PI 3-kinase protein levels were evaluated by densitometry of western blotting. Figure 5A shows the substantially higher intensity of phosphorylated Akt (phospho-Akt), phosphorylated ERK-1/2 (phospho-ERK-1/2), and PI 3-kinase protein and a four-fold reduction of the Bax protein in the AdMK group compared with the AdLacZ group with similar expression of GAPDH in both groups. AdMK may promote anti-apoptosis in the heart after MI by increasing Bcl-2 protein and a four-fold reduction of the Bax protein in the AdMK group compared with the AdLacZ group. This is consistent with other growth factors, gene therapy with overexpressed MK has never been reported previously. In this study, we have shown that adenoviral gene transfer of MK has strong effects on the infarcted heart in order to explain the significant amelioration of ischemic cardiomyopathy by AdMK treatment normalized the HW/BW ratio after MI compared with the AdLacZ group to values not different from sham-operated animals. Haemodynamically, systolic and diastolic functions in the AdMK group were better than in the AdLacZ group, although still worse than in sham-operated animals. Nevertheless, most parameters in the AdMK group were much closer to those in the sham group without MI than to those in the AdLacZ group with MI.

3.7 Anti-apoptotic effects of the Bcl-2 family

To elucidate the mechanism of anti-apoptosis, Bcl-2 and Bax protein levels were evaluated by western blots as described in the previous section. Figure 6A shows representative bands of increased Bcl-2 and decreased Bax proteins in the AdMK group compared with the AdLacZ group with similar expression of GAPDH in both groups. Figure 6B shows the quantified changes with doubling of Bcl-2 protein and a four-fold reduction of the Bax protein in the AdMK group. AdMK may promote anti-apoptosis in the heart after MI by upregulating the anti-apoptotic Bcl-2 protein and by downregulating the pro-apoptotic Bax protein.

4. Discussion

In this study, we have presented long-term effects of MK gene transfer in a setting of chronic MI. As a result from infarct reduction, LVEDD and LVEF and decreased Bax activity and may constitute the key factor by which AdMK ameliorates cardiac dysfunction after MI. Mitigation of ischemic wall stress.18 Infarct expansion also causes the deformation of the infarcted heart in order to explain the significant amelioration of ischemic cardiomyopathy by AdMK treatment normalized the HW/BW ratio after MI compared with the AdLacZ group to values not different from sham-operated animals. Haemodynamically, systolic and diastolic functions in the AdMK group were better than in the AdLacZ group, although still worse than in sham-operated animals. Nevertheless, most parameters in the AdMK group were much closer to those in the sham group without MI than to those in the AdLacZ group with MI.

### Table 2: Haemodynamic and morphological data at 6 weeks after MI

<table>
<thead>
<tr>
<th></th>
<th>Sham-operated rats (n = 8)</th>
<th>AdLacZ rats (n = 12)</th>
<th>AdMK rats (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM + AdLacZ (n = 4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR, b.p.m</td>
<td>266 ± 26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>2.1 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dP/dt;max, mmHg/s</td>
<td>5802 ± 248</td>
<td></td>
<td></td>
</tr>
<tr>
<td>−dP/dt;max, mmHg/s</td>
<td>−5737 ± 757</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW, g (pre-operation)</td>
<td>276 ± 13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW, g (post-treatment)</td>
<td>385 ± 16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HW, mg</td>
<td>1201 ± 127</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HW/BW, mg/g</td>
<td>3.1 ± 0.24</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Sham + AdLacZ and sham + AdMK are used as the same in Table 7. HR, heart rate; LVEDP, left ventricular end-diastolic pressure; dP/dt;max and −dP/dt;max, maximal and minimal first derivatives of LV pressure; BW, body weight; HW, heart weight. Values are mean ± SEM.

*P < 0.001 vs. sham-operated rats.
**P < 0.001 vs. AdLacZ rats.
***P < 0.001 vs. AdMK rats.

4.1 Adenoviral gene transfer of MK

Adenoviral gene transfer with intracoronary or intramyocardial injections has been used safely in clinical trials.12–16 Local gene transfer of adenoviral vectors has some advantages in terms of the duration of the effects, the avoidance of systemic exposure, and the simplicity of delivery to target tissues compared with protein delivery. Different from other growth factors, gene therapy with overexpressed MK has never been reported previously. In this study, we have shown that adenoviral gene transfer of MK has strong effects on the infarcted heart comparable to previously reported MK protein therapy.5,6

4.2 Temporal aspects of adenoviral gene transfer of MK

In MI rats, cardiac dysfunction and chamber dilatation develop within 1 week after MI, and significant and rapid progressive LV dysfunction follows between the first and second month.17 In addition, infarct expansion occurs within hours of myocyte injury, leading to wall thinning and ventricular dilatation and elevation of diastolic and systolic wall stress.18 Infarct expansion also causes the deformation of the border-zone and remote normal myocardium, which alters the normal Frank–Starling relationship. The BWt was larger in the AdMK group compared with the AdLacZ group. This is consistent with improved cardiomyocyte survival promoted by increased Bcl-2 and decreased Bax activity and may constitute the key factor by which AdMK ameliorates cardiac dysfunction after MI. Adenovirus encoding MK apparently worked for at least 8 weeks after injection in the infarcted heart in order to explain the significant amelioration.
of LV dysfunction and remodelling at 4 weeks and even more obvious at 6 weeks after MI.

4.3 Reduction of fibrosis
Deposition of types I and III collagen occurs in infarcted and non-infarcted myocardium when intercellular signalling is potentiated by extensive myocardial necrosis. MK has been reported to upregulate the expression of transforming growth factor-β1 and to stimulate collagen production. The border-zone wall composed of this thick fibrosis and the residual myocardium through anti-apoptosis in the AdMK group may retain LV geometry after MI. Thus AdMK may be correlated with the attenuation of tissue deformation in the non-infarct area and the prevention of collagen deposition in this area.

Increased myocardial fibrosis directly links to abnormalities in diastolic function and myocardial stiffness. In our study, collagen deposition in non-infarct area was reduced in the AdMK group compared with the AdLacZ group. The reduction of collagen deposition in non-infarcted myocardium in the AdMK group may result in the preservation of LV diastolic dysfunction after MI.

4.4 Stimulation of angiogenesis and anti-apoptosis
MK has been reported to enhance tumour growth, endothelial cell proliferation, and vascular density. MK expression leads to the release of endothelial growth-stimulating factors from transfected cells, which confers an angiogenic activity on these cells, and this activity is critical for promoting new vessel formation in the heart.
angiogenesis enhances both tumour growth and vascular density. Collagen gel including MK enhances neovascularization in the infarct and border-zone area after MI. MK also has anti-apoptotic effects via the activation of ERK, and MK also upregulates an anti-apoptotic factor, Bcl-2 in tumour cell lines and in cultured cardiomyocytes. The enhancement of neovascularization and anti-apoptosis in the heart after MI by MK gene transfer may ameliorate cardiac dysfunction and prevent remodelling. Angiogenesis is particularly effective in the early post-infarction period when significant areas of border-zone myocardium are viable but hypokinetic or akinetic because of coronary ischemia. Also in the late remodelling phase neovascularization may permit collateral flow and attenuate the progression of late remodelling. Correspondingly, the blockade of the activation of apoptosis following myocardial injury prevents cell loss and preserves myocardial geometry and function.

4.5 Molecular mechanisms

In the present study, we elucidate the molecular biological background of angiogenic and anti-apoptotic effects of MK gene transfer. The PI 3-kinase/Akt signalling pathway has a potential role in inhibition of cardiomyocyte apoptosis and induces vascularization of myocardium after MI. The ERK-1/2 MAP kinase pathway is also a key signalling cascade in the modulation of angiogenesis and p38 MAPK/ERK-1/2 signalling is cardioprotective by suppression of apoptosis. In the Bcl-2 family, the balance in expression between Bcl-2 and Bax has been suggested to be pivotal for apoptotic cell death. In the AdMK group, the levels of phospho-Akt, phospho-ERK, and PI 3-kinase were increased compared with the AdLacZ group. (A) Representative bands of phosphorylated Akt, total Akt, phosphorylated ERK-1/2, total ERK-1/2, PI 3-kinase, and GAPDH are demonstrated. (B) The bar graphs of phosphorylated Akt and ERK show the ratio of the phosphorylated form of Akt or ERK to total Akt or ERK as relative intensity of the AdMK group compared with the AdLacZ group. The bar graphs of PI 3-kinase represents the relative intensity in the AdMK group compared with the AdLacZ group. Values are mean ± SEM. #p < 0.05, †p < 0.01 vs. AdLacZ (n = 5 per each).
3-kinase were significantly elevated compared with the AdLacZ group. Upregulation of Bcl-2 and downregulation of Bax shifted another anti-apoptotic balance in a favourable direction in the AdMK group.

4.6 Limitation and future aspects
MK promotes tumour angiogenesis as well as ischemic myocardial angiogenesis. Transient local administration, rather than systemic administration, may therefore limit the risk of carcinogenesis. But the application of angiogenic factors for the treatment of ischemic heart disease needs further thorough consideration of risk/benefit balance, in particular, in patients with carcinomas. In addition, adenovirus provokes an immune response and a significant inflammatory response apart from the induction of apoptosis. Adenoviral vectors themselves should be improved for the purpose of delivering genes safely by percutaneous catheter-based myocardial injection or intracoronary injection.

In conclusion, we have demonstrated in this study that overexpression of MK improves long-term LV dysfunction and limits remodelling after MI through angiogenic and anti-apoptotic effects. MK gene therapy may have the potential of becoming a therapeutic strategy in ischemic heart disease and ischemic heart failure in the near future.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

Acknowledgement
We thank T. Koike and M. Hojo for technical assistance.

Conflict of interest: none declared.

Funding
This work was supported by the Ministry of Education, Culture, Sports, Sciences and Technology, Japan.

References
Midkine prevents remodelling after myocardial infarction

22. Choudhuri R, Zhang HT, Donini S, Ziche M, Bicknell R. An angiogenic role for the
18. Sutton MG, Sharpe N. Left ventricular remodeling after myocardial infarction: Patho-
15. Losordo DW, Vale PR, Hendel RC, Asahara T, Isner JM, Kunts RE
18. Sutton MG, Sharpe N. Left ventricular remodeling after myocardial infarction: Patho-
15. Losordo DW, Vale PR, Hendel RC, Asahara T, Isner JM, Kunts RE
18. Sutton MG, Sharpe N. Left ventricular remodeling after myocardial infarction: Patho-
15. Losordo DW, Vale PR, Hendel RC, Asahara T, Isner JM, Kunts RE