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

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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 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.

Keywords

Gene transfer • Coronary disease • Infarction • Haemodynamics • Remodelling

1. Introduction

Midkine (MK) is a heparin-binding growth factor involved in neural survival, carcinogenesis, and tissue repair.1 The mobilization of inflammatory cells is closely related to the angiogenic efficacy of MK.2 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. We previously 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 anaesthetized 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.

2.3 Echocardiography
Transthoracic echocardiography was performed to study cardiac functional parameters with a Nemio 20 (Toshiba Medical) before and after MI as described previously. Each rat was anaesthetized 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. To 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-vWF 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, Nicon) 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 blots at 1 week after MI as previously described. Briefly, LV myocardial homogenates were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis on a 10–12.5% polyacrylamide gel and proteins were electrophoretically transferred to 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 echocardiogra-
phy. 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
(Figure 1A, red arrows). They were seen throughout the injected
areas of the rat LV wall. In western blotting followed by IP with pro-
teins from ventricular homogenates, the LV myocardium of the AdMK
group showed substantial overexpression of MK protein as strong as
in the kidney, which possesses abundant endogenous MK protein. In
contrast, the expression of MK protein was not detected in the
AdLacZ group (Figure 1B). Thus AdLacZ and AdMK were successfully
injected into the LV myocardium. In addition, the transferred adenovi-
al genes were effectively expressed in in vivo rat hearts. To examine
the long-term MK expression by adenoviral gene transfer, we also
analysed the expression of MK at 1, 2, 4, 6, 8, and 10 weeks after
gene transfer in sham-operated rats by western blots. Figure 1C
shows the time course of MK expression after AdMK injection. The
expression of MK declined gradually from 1 week on. It was weakly
detectable at 8 weeks and it was no longer detectable at 10 weeks
after adenoviral injection (Figure 1D).

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%. Echocar-
diography was repeated at 4 weeks after MI. There were no differ-
ences 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 con-
trastile 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

Figure 1 In vivo adenoviral infection and gene expression at 1
week after injection into LV myocardium of rat heart without MI.
(A) Representative X-gal staining with counterstaining neutral red
of heart section in the AdLacZ group. Red arrows point to the
cells stained blue which express β-galactosidase gene in the injected
LV myocardium. Scale bar represents 100 μm. (B) Immuno-blots
with IP for MK protein (13 kDa) were performed by using ventricular
homogenates in the injected areas with AdLacZ or AdMK and kidney
homogenates. (C) Western blot of MK protein was performed by
using the injected myocardium in sham-operated rats. Representa-
tive bands of MK and GAPDH at 1, 2, 4, 6, 8, 10 weeks after
AdMK injection are demonstrated. (D) The graph shows the
expressions of MK at each week. They were represented with relative
intensity to 1 week.
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\text{max}) and lower minimum dP/dt (−dP/dt\text{min}) compared with the AdLacZ group (Table 2). The AdMK group also had lower LVEDP compared with the AdLacZ group. The dP/dt\text{max} − dP/dt\text{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.
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 in the AdMK group compared with the AdLacZ group while total Akt, total ERK-1/2, and GAPDH showed similar expression in both groups. For phosphorylated Akt, phosphorylated ERK-1/2, and PI 3-kinase, the levels were increased by 77, 43, and 136%, respectively, in the AdMK group compared with the AdLacZ group (Figure 5B). AdMK enhances angiogenesis (Figure 4), and this angiogenic action may thus be mediated by PI3K/Akt and ERK-1/2 pathways (Figure 5B).

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 LVESD in the AdMK group were smaller than those in the AdLacZ group, and the larger BWT in the AdMK group limited the deterioration of LVEF and SF after MI. Mitigation of ischemic cardiomypathy 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.

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 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.

Figure 3 Picosirius red staining at 6 weeks after MI to quantify collagen deposition. (A) Representative cross-section of an infarcted heart from the AdLacZ group. (B) Representative cross-section of an infarcted heart from the AdMK group. Scale bars represent 5 mm. (C) Representative border-zone area of infarcted heart from the AdLacZ group. (D) Representative border-zone area of infarcted heart from the AdMK group. Scale bars represent 200 μm. (E) Representative non-infarct area of an MI heart from the AdLacZ group. (F) Representative non-infarct area of an MI heart from the AdMK group. Scale bars represent 50 μm. (G) Collagen deposition (%) in the non-infarct areas from the AdLacZ and AdMK groups. Values are mean ± SEM. *P < 0.01 vs. AdLacZ (n = 12 per each).

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
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 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.

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