Antibody against interleukin-6 receptor attenuates left ventricular remodelling after myocardial infarction in mice

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Aims

The plasma level of interleukin-6 (IL-6) has been reported to be associated with left ventricular (LV) remodelling after myocardial infarction (MI). The present study was designed to examine whether anti-IL-6 receptor antibody (MR16-1) prevents the development of LV remodelling after MI.

Methods and results

Balb/c male mice were subjected to MI by ligating the left anterior descending coronary artery. The mice were then treated with an intraperitoneal injection of MR16-1 (500 μg/body) or control IgG. MR16-1 decreased the myocardial myeloperoxidase activity and monocyte chemoattractant protein-1 concentration in the infarct region, concomitant with decreases in neutrophil and macrophage infiltration 3 days after ligation, while infarct size was comparable between the control IgG- and MR16-1-treated mice. At 7 days after ligation, MR16-1 significantly suppressed matrix metalloproteinase-2 activity in the infarct region. Furthermore, the MR16-1-treated mice demonstrated a reduction in LV dilatation and an improvement in LV contractile function compared with the control IgG-treated mice at 7 and 28 days after surgery, leading to an improvement in survival rate (80.6 vs. 59.5%, P < 0.05) at 28 days after surgery. The beneficial effects of MR16-1 were accompanied by histological suppression of cardiomyocyte hypertrophy and interstitial fibrosis in the non-infarct region.

Conclusion

Administration of MR16-1 after MI suppressed myocardial inflammation, resulting in the amelioration of LV remodelling. Neutralization of the IL-6 receptor is a potentially useful strategy for protecting hearts from LV remodelling after MI.

Keywords

Interleukin-6 • Myocardial infarction • Left ventricular remodelling

1. Introduction

Acute myocardial infarction (MI) is one of the most common cardiovascular diseases and leads to left ventricular (LV) dilatation, resulting from expansion of the infarct region and eccentric hypertrophy of the non-infarct region. These structural alterations, which are known as ventricular remodelling, cause impairment of cardiac function following heart failure.¹ Inhibition of the renin–angiotensin system and blockade of the β receptor have been reported to attenuate LV remodelling and improve mortality and morbidity in patients with MI in novel clinical trials.²–⁴ However, these therapeutic approaches did not suppress the progression of LV remodelling sufficiently.

The plasma levels of pro-inflammatory cytokines, such as interleukin-6 (IL-6), TNFα, and IL-1β, are elevated after MI.⁵–⁷ It is becoming increasingly apparent that these cytokines play an important role in the modulation of the inflammatory response after MI and are associated with the subsequent LV remodelling.⁸–¹² Among these cytokines, the peak plasma IL-6 level correlates positively with the area under the curve of creatine kinase MB mass¹³ and is a powerful predictor of LV remodelling.¹⁴ Not only the circulating level of IL6, but also the myocardial IL-6 level is elevated after MI. Myocardial IL-6 transcription shows robust up-regulation within several hours in both infarct and non-infarct areas.¹⁵,¹⁶ IL-6 activates cells by binding to the membrane-bound receptor (mIL-6R) and the soluble form of the IL-6 receptor (sIL-6R), inducing

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Homodimerization of signal-transducing gp130 molecules. Homodimerization of gp130 leads to the activation of intracellular signalling cascades including the Janus kinase/signal transducer and activator of transcription (JAK/STAT), Ras/mitogen-activated protein kinase (MAPK), and phosphatidylinositol-3 kinase pathways. In the infarcted myocardium, the major IL-6R-expressing cells are neutrophils and monocytes. Moreover, the plasma level of sIL-6R in patients with acute MI is elevated, which induces the subsequent activation of gp130-expressing cells, including cardiac myocytes, by IL-6/sIL-6R complex.

Therefore, IL-6 may play an important role in myocardial inflammation after MI. However, previous studies have demonstrated the pathophysiological significance of IL-6R in MI. In the present study, we examined the effects of IL-6R neutralization on post-infarct LV remodelling.

2. Methods

All animals were handled 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). The protocol was approved by the Bioethics Committee of Kyoto Pharmaceutical University, and approval reference number is 053001.

2.1 MI model and drug administration

Male Balb/c mice of 8- to 12-week old and 25–30 g body weight were anaesthetized with diethyl ether, before being intubated and ventilated with a rodent respirator under isoflurane anaesthesia. After thoracotomy, MI was induced by ligation the left anterior descending coronary artery (LAD) with a 6-0 nylon suture. Successful ligation of the LAD was verified by the appearance of cyanosis of the anterior LV. The chest wall and skin were then closed with 6-0 silk sutures. The sham operation was performed in the same manner but without ligation. The MI animal was randomly divided into two groups and were intraperitoneally injected with rat anti-mouse IL-6R monoclonal antibody (MR16-1; 500 μg/body). The specificity and blocking ability of this monoclonal antibody were confirmed in previous studies.

2.2 Echocardiographic studies

At 7 and 28 days after surgery, transthoracic echocardiography was performed with a 15-MHz sector scan probe (SONOS5500, Phillips Medical Systems Japan, Tokyo, Japan). The LV end-diastolic and end-systolic diameters (LVDd and LVDs) were measured on the M-mode tracings. Fractional shortening (FS) was calculated as FS (%) = 100 × [(LVDd – LVDs)/LVDd].

2.3 Morphological determination of infarct size

At 3 days after MI, the heart was removed and sliced transversely from the base to apex into five sections. The tissue slices were then incubated for 15 min in a 0.2 mol/L Tris–HCl buffer (pH 8.0) containing 1.0% triphenyl tetrazolium chloride (TTC) to identify infarcted tissue. The amount of infarcted tissue was expressed as a percentage of the total LV area.

2.4 Histological and immunohistological analyses

Histological analysis was performed as described previously with some modifications. Three or 28 days after MI, the heart was excised and fixed in buffered formalin. The LV was then cut into three transverse sections parallel to the atrioventricular groove (apex, middle, and base sections) and embedded in paraffin for histological studies. The sections were cut into 4 μm slices and stained with haematoxylin–eosin (HE). Masson’s trichrome, and 0.1% Sirius red. Cardiac myocyte size in the remote zone was assessed by cross-sectional area measurement of myocytes at 28 days after MI using Masson’s trichrome-stained sections from the middle LV. Remote zone fibrosis was analysed semi-quantitatively by determination of collagen deposition at 28 days after MI using Sirius red-stained sections from the middle LV. Five independent fields of non-infarcted myocardium from each mouse were photographed with an optical microscope system (Olympus IX71). The images were analysed using Scion Image, and myocyte cross-sectional area and the percentage area of interstitial fibrosis were calculated.

To examine myocyte apoptosis, tissue sections from the middle LV at 3 days after MI were stained by the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) method. TUNEL-positive nuclei were visualized with 3,3′-diaminobenzidine. Then, to detect myocytes, the sections were stained with anti-desmin antibody (MONOSAN, The Netherlands) and biotinylated goat anti-rabbit IgG. Subsequently, the sections were incubated with alkalinephosphatase-conjugated streptavidin, and alkalinephosphatase activity was visualized with 5-bromo-4-chloro-3-indol phosphate/nitroblue tetrazolium. Finally, the sections were counterstained with haematoxylin to visualize the nuclei. The nuclei located in the myocytes were recognized as myocyte nuclei, and we calculated the proportion of TUNEL-positive myocytes nuclei in the ischaemic border region using a light microscope. The ischaemic border region was defined as a 0.1 mm region bordering the necrotic region in continuous sections stained with HE.

To detect macrophage infiltration, tissue sections from the middle LV at 3 days after MI were stained immunohistochemically with rat anti-mouse macrophage antibody (Mac3, BD Biosciences, CA, USA). For the negative control, non-immunized rat IgG was used. The sections were incubated with biotinylated anti-rat IgG and covered with streptavidin peroxidase, and then peroxidase activity was visualized with 3,3′-diaminobenzidine tetrahydrochloride.

2.5 Myocardial myeloperoxidase activity

Neutrophil accumulation in the myocardium was assessed by determining the activity of myeloperoxidase (MPO), an enzyme specific to neutrophils. At 3 days after MI, the hearts were flushed with cold phosphate-buffered saline (PBS) to remove blood from the vasculature, and the LV tissues were divided into infarct and non-infarct regions. The tissues were then homogenized and sonicated in 50 mmol/L potassium phosphate buffer at pH 6.0 containing 0.5% hexadecyltrimethylammonium bromide. The resulting homogenates were centrifuged at 12 000 g for 15 min at 4°C. The supernatants were collected and placed on ice. The supernatants were filtered through a 0.45-μm filter, and 0.167 mg/mL o-dianisidine dihydrochloride and 0.0005% H2O2 in 50 mmol/L potassium phosphate buffer at pH 6.0 were added. The change in absorbance was measured with a spectrophotometer at 450 nm, and activity was calculated with an extinction coefficient for o-dianisidine at 460 nm of 1.13 × 104 mol/L−1 cm−1. The data are expressed as MPO activity per gram tissue.

2.6 Enzyme-linked immunosorbent assay

To examine tissue monocyte chemoattractant protein-1 (MCP-1) (at 3 days after MI) and IL-6 concentrations (on the day before MI and at 2.5 days after MI, hearts were extracted and divided into infarct and non-infarct regions and homogenized with lysis buffer (2 × PBS, 1% NP-40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS), 1 mmol/L PMSF, and 1% protease inhibitor cocktail (Nacalai Tesque Inc., Kyoto, Japan); pH 7.5). The homogenate were then centrifuged at 8000 g for 10 min at 4°C, and the supernatants were collected and stored at −80°C until enzyme-linked immunosorbent assay (ELISA). The concentrations of MCP-1 and IL-6 were measured using specific ELISA kits (MCP-1: BD Biosciences; IL-6: Amersham Biosciences, UK).
2.7 Gelatin zymography

The matrix metalloproteinase (MMP) activity in cardiac tissue was measured by gelatin zymography at 7 days after MI. Samples containing equal amounts of protein were mixed with an equal volume of 2× loading buffer (0.125 mmol/L Tris–HCL, 20% glycerol, 4% SDS, and 0.005% bromophenol blue; pH 6.8) and separated in gelatin zymography gel by electrophoresis. Then, the gels were incubated in 2.5% Triton X-100 for renaturation for 30 min and incubated for 12 h at 37°C in a developing buffer containing 50 mmol/L Tris, 0.2 mol/L NaCl, 5 mmol/L CaCl2, and 0.02% Brace-35. The gels were then stained with 0.5% Coomasie Blue (40% methanol and 10% acetic acid) for 2 h, and destained in 50% methanol and 10% acetic acid. The areas containing gelatinase activity appeared as clear bands against a dark blue background.

2.8 Statistical analysis

All values are expressed as the mean ± SEM. Survival analysis was performed by the Kaplan–Meier method, and differences in survival were tested using the log-rank test. Other data were analysed by one-way ANOVA combined with Fisher’s multiple comparison test (Figures 1B and 3–6; Table 1) or the Student’s t-test (Figure 2). P-values < 0.05 were considered significant.

3. Results

3.1 Survival study

Early operative mortality (within 24 h) was comparable between the groups (16.0 ± 1.7% for the control IgG-treated MI group vs. 18.0 ± 0.8% for the MR16-1-treated MI group, P = n.s.). The 73 animals that survived for 24 h after surgery were included in the Kaplan–Meier survival analysis. The survival rate of the MI mice at 28 days was significantly higher in the MR16-1-treated MI group (59.5% for the control IgG-treated MI group vs. 80.6% for the MR16-1-treated MI group, P < 0.05, Figure 1A). All mice that died were confirmed to have suffered MI by post-mortem examination. Death was commonly attributed to congestive heart failure or arrhythmia. In addition, the rate of LV rupture was similar between the groups (2.4% for the control IgG-treated MI group vs. 3.2% for the MR16-1-treated MI group, P = n.s.).

3.2 Organ weights and echocardiography

At 7 and 28 days after ligation, body weight was comparable among all groups (Table 1). At 7 days after ligation, the ratio of LV weight to body weight was significantly increased in both the control IgG- and the MR16-1-treated MI groups, and the difference between them was not significant. In contrast, the ratio of lung weight to body weight was significantly increased in the control IgG-treated MI group, and this increase was attenuated in the MR16-1-treated MI group.

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**Table 1 Physical characteristics and echocardiographic data at 7 and 28 days after coronary ligation (n = 6–10)**

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Control</th>
<th>MR</th>
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<tr>
<td><strong>7 days after ligation</strong></td>
<td></td>
<td></td>
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<tr>
<td>BW (g)</td>
<td>21.7 ± 0.6</td>
<td>20.5 ± 0.5</td>
<td>20.7 ± 0.8</td>
</tr>
<tr>
<td>LV Wt/BW (mg/g)</td>
<td>3.50 ± 0.11</td>
<td>4.71 ± 0.17</td>
<td>4.46 ± 0.19</td>
</tr>
<tr>
<td>Lung Wt/BW (mg/g)</td>
<td>5.63 ± 0.33</td>
<td>8.95 ± 0.75</td>
<td>7.20 ± 0.45*</td>
</tr>
<tr>
<td>LVDd (mm)</td>
<td>2.82 ± 0.04</td>
<td>4.27 ± 0.05†</td>
<td>3.74 ± 0.13‡,*</td>
</tr>
<tr>
<td>FS (%)</td>
<td>46.9 ± 2.4</td>
<td>11.7 ± 0.3‡</td>
<td>18.3 ± 1.2†,‡,*</td>
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|                  |            |            |            |
| **28 days after ligation** |            |            |            |
| BW (g)           | 26.9 ± 0.6 | 27.6 ± 0.7 | 27.2 ± 0.8 |
| LV Wt/BW (mg/g)  | 3.85 ± 0.19| 5.15 ± 0.29†| 4.87 ± 0.25‡|
| Lung Wt/BW (mg/g)| 6.15 ± 0.18| 12.20 ± 1.86#| 7.85 ± 1.45‡|
| LVDd (mm)        | 3.01 ± 0.09| 4.66 ± 0.13‡| 4.16 ± 0.18‡,*|
| FS (%)           | 45.2 ± 3.1 | 10.7 ± 0.9‡ | 18.2 ± 2.7†‡,*|

**Note:** BW, body weight; LV Wt, LV weight; lung Wt, lung weight; LVDd, left ventricular end-diastolic diameter; FS, fractional shortening.

*P < 0.05 vs. sham.
†P < 0.01 vs. sham.
‡P < 0.05 vs. control.
after MI. IL-6 levels in this region gradually increased from 21 to 28 days surgery and quickly fell back to the baseline level. However, the myocardial IL-6 content had increased 1.8-fold at 1 day after baseline by 3 days. On the other hand, in the non-infarct region, at 1 day after surgery (up to 3.3-fold) and had fallen to near to the baseline level. The size of the infarct area relative to the LV myocardial area (n = 6). The percentage of TUNEL-positive myocyte nuclei in the ischaemic border area (n = 7).

3.3 Time course of myocardial IL-6 level
The evolution of the myocardial level of IL-6 after surgery is shown in Figure 1B. In the infarct region, the level of IL-6 was drastically elevated at 1 day after surgery (up to 3.3-fold) and had fallen to near to the baseline by 3 days. On the other hand, in the non-infarct region, the myocardial IL-6 content had increased 1.8-fold at 1 day after surgery and quickly fell back to the baseline level. However, the IL-6 levels in this region gradually increased from 21 to 28 days after MI.

3.4 Infarct size and the proportion of apoptotic myocytes
Infarct size, as determined by morphometric TTC analysis 3 days after ligation, was comparable (47.1 ± 6.0 vs. 48.6 ± 5.5%, P = n.s.) between the control IgG- and MR16-1-treated MI groups (Figure 2A and B). To visualize apoptosis in situ, the TUNEL method was employed in myocardial specimens at 3 days after ligation. TUNEL-positive myocytes were mainly observed in the ischaemic border region, and the percentage of apoptotic myocytes in this region was also comparable between the groups (3.0 ± 0.2% in the control IgG-treated MI group and 2.7 ± 0.3% in the MR16-1-treated MI group, P = n.s.; Figure 2C and D).

3.5 Myocardial neutrophil infiltration and MPO activity
Three days after ligation, abundant polymorphonuclear leucocyte (neutrophils) infiltration was exhibited in the infarct zone of the control IgG-treated group (Figure 3A and C), whereas MR16-1 markedly suppressed neutrophil infiltration (Figure 3B and D). MPO is released from neutrophils, and its activity is a marker of tissue neutrophil infiltration. Moreover, MPO has been reported to have a profound adverse effect on LV remodelling. At 3 days after MI, the myocardial MPO activity in the infarct region was increased seven-fold compared with that in the sham-operated hearts, whereas MR16-1 significantly reduced MPO activity in the infarct region (Figure 3E).

3.6 Myocardial MCP-1 content and macrophage infiltration
MCP-1 is one of the major recruiters of monocytes and is a critical factor in LV remodelling after MI. In the present model, the infarcted myocardia contained a 10-fold excess of MCP-1 at 3 days after surgery in the control IgG-treated MI group, whereas MR16-1 attenuated the MCP-1 increase in this region. In the non-infarct region, the MCP-1 concentration was significantly increased (up to 4.4-fold) in the control IgG-treated group, but not in the MR16-1-treated group (Figure 4A). Moreover, numerous macrophages, as identified by immunohistochemical staining of Mac3, had infiltrated into the ischaemic border region at 3 days after surgery in the control IgG-treated MI group (Figure 4B). In contrast, there was a marked reduction in the number of infiltrated macrophages in the MR16-1-treated group (Figure 4C).

3.7 Myocardial MMP-2 activity
At 7 days after surgery, the myocardial MMP-2 activity increased three-fold in the non-infarcted myocardia and seven-fold in the infarcted myocardia in the control IgG-treated MI group compared with the sham-operated hearts as revealed by in vitro zymography (Figure 5). The MR16-1-treated hearts exhibited significant attenuation of MMP-2 gelatinolytic activity in the infarcted myocardium.

3.8 Histomorphometry
Transverse mid-LV sections stained with Masson’s trichrome are shown in Figure 6A–E. Consistent with the echocardiographic data, the MR16-1-treated MI mice revealed a significantly smaller LV chamber diameter compared with the control IgG-treated MI mice (Figure 6A and B). Photomicrographs of the LV sections showed an increased myocyte cross-sectional area in the non-infarct region in the control IgG-treated MI group, and this myocyte hypertrophy was significantly attenuated by MR16-1 (Figure 6C–E and I). In addition, the incidence of interstitial fibrosis, as determined by Sirius red staining, was obviously increased in the remote areas of the control IgG-treated MI group, and this increase was suppressed by treatment with MR16-1 (Figure 6F–H and J).
4. Discussion

The present study is the first work to demonstrate that inhibition of IL-6R has beneficial effects on the development of LV remodelling after MI. Administration of the selective IL-6R antagonist MR16-1 reduces leucocyte and macrophage infiltration and attenuates MMP activation, leading to a marked improvement in LV dilatation, contractile dysfunction, and lifespan.

There are increasing numbers of studies demonstrating that increasing the expression of pro-inflammatory cytokines exacerbates myocardial inflammatory damage, leading to progressive impairment of cardiac function.8–10,27–30 The present results are consistent with those of prior studies. Pro-inflammatory molecules are known to interact with each other. IL-6 induces the expression of intracellular adhesion molecule-1, resulting in the subsequent adhesion of neutrophils15,31 and an increase in the neutrophil-mediated tissue MPO level. MPO is a major enzymatic source of leucocyte-generating oxidants and causes concurrent cytotoxicity of cardiac myocytes.32 As expected, MPO-null mice experienced suppression of adverse LV dilatation and improvement of LV function.25,26 Moreover, increases in MCP-1 expression and monocyte infiltration are reported to be associated with IL-6 receptor activation,33,34 and MCP-1 is also an exacerbating factor in LV remodelling after MI.8–10 In addition, enhancement of myocardial MCP-1 expression promoted the induction of MMP, especially of MMP-2 and MMP-9, in an experimental
MMP-9 prevents post-infarction LV remodelling. Thus, the inhibition of the IL-6R attenuates the vicious circle of pro-inflammatory amplification, including that of MPO, MCP-1, and MMP, and the development of LV remodelling.

On the other hand, cytokine-mediated post-infarct inflammation also initiates wound healing. Neutralization of interleukin-1β in the acute phase of MI leads to poor wound healing, resulting in the promotion of cardiac rupture and the progression of LV dilatation. Defective infarct healing leads to inadequate extracellular matrix (ECM) accumulation, which results in an increased risk of cardiac rupture. Cardiac rupture is one of the most severe complications after MI. In the present study, MR16-1 did not affect the incidence of LV rupture. In this model, MR16-1 obviously suppressed the acute phase of inflammation, leading to delayed wound healing and ECM accumulation. On the other hand, MR16-1 suppressed MMP-2 activation, which would suppress ECM degradation. In the present study, the infarct wall thinning detected with echocardiography was similar in both the control IgG- and MR16-1-treated MI mice at 7 days after MI (0.41 ± 0.04 mm in the control IgG-treated MI group and 0.48 ± 0.08 mm in the MR16-1-treated MI group, P = n.s.).

Taken together, it is suggested that the accumulation and degradation of ECM protein were balanced in the present model. Furthermore, we examined the possibility that delayed wound healing prolonged the progression of LV remodelling. At 2 months after MI, echocardiography showed continuous attenuation of LV dilatation and improvement in LV function after MR16-1 treatment (data not shown). Therefore, these agents might suppress LV remodelling rather than just delay the remodelling process.

In the present study, we injected MR16-1 intraperitoneally once after MI. It is very important to know how long MR16-1 can maintain its effects. Previously, Okada et al. reported that the half-life of MR16-1 was 3 days in intraperitoneally injected mice. In the present model, the myocardial IL-6 level was significantly increased in both infarct and non-infarct regions 1 day after MI, but had decreased in both regions at 3 days after MI. In addition, it has been reported that the expression of IL-6 receptors is up-regulated after 24 h of sustained ischaemia. MR16-1 inhibits this acute increase in IL-6 signalling, leading to the suppression of the subsequent inflammation. Then, the myocardial IL-6 levels increased again in the non-infarct region at 21–28 days after MI. MR16-1 suppressed cardiac fibrosis and myocyte hypertrophy in the non-infarct region at 28 days after MI. IL-6 has been reported to be an inducer of myocyte hypertrophy; however, in view of its half-life, it is unlikely that MR16-1 directly inhibited the late increase in IL-6 signalling in the non-infarct region. Thus, in the present model, the suppression of the initial inflammatory response might have ameliorated LV remodelling, including myocyte hypertrophy.

Two previous reports examined therapeutic interventions targeting IL-6. First, Fuchs et al. reported that targeted deletion of the IL-6 gene did not affect infarct size or LV remodelling using a permanent coronary occlusion model. In their report, as well as our’s, the authors examined the effect of IL-6 signal deletion on MI; however, the results were not similar. The reason for this discrepancy is not clear, but as the authors mentioned, compensatory activation of other IL-6 family proteins might have contributed to their genetic deletion model. Secondly, Matsushita et al. reported that combination therapy involving IL-6 and sIL-6R inhibited cardiomyocyte apoptosis and reduced infarct size. The IL6/sIL-6R complex is increased after
IL-6 family proteins such as leukaemia inhibitory factor, whose receptor is abundantly expressed in myocytes and activates signalling pathway that is also activated by IL-6/sIL-6R complex, is increased after MI. The lack of effect of MR16-1 on myocyte apoptosis suggests that the role of IL-6/sIL-R complex had little to do with protecting hearts from LV remodelling after MI.

In conclusion, a single injection of MR16-1 after MI suppressed myocardial inflammation, resulting in the amelioration of LV remodelling. Inhibition of the IL-6 signalling pathway may be a novel strategy for protecting hearts from LV remodelling after MI.

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