Atorvastatin enhances interleukin-10 levels and improves cardiac function in rats after acute myocardial infarction

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ABSTRACT
LV (left ventricular) remodelling is the basic mechanism of HF (heart failure) following MI (myocardial infarction). Although there is evidence that pro-inflammatory cytokines [including TNF-α (tumour necrosis factor-α) and IL-6 (interleukin-6)] are involved in the remodelling process, only little is known about the role of anti-inflammatory cytokines, such as IL-10. As accumulating evidence has revealed that statins possess anti-inflammatory properties, the aim of the present study was to elucidate the effect of atorvastatin on the modulation of the anti-inflammatory cytokine IL-10 and its effect on LV function in rats with HF subsequent to MI. Rats with MI, induced by permanent LAD (left anterior descending) branch coronary artery ligation, were treated for 4 weeks with atorvastatin (10 mg·kg⁻¹·day⁻¹ via oral gavage) starting on the first day after induction of MI. Cardiac function was assessed by echocardiography and cardiac catheterization 4 weeks after MI induction. Membrane-bound and soluble fractions of TNF-α, IL-6 and IL-10 protein, the TNF-α/IL-10 ratio, serum levels of MCP-1 (monocyte chemoattractant protein-1) as well as myocardial macrophage infiltration were analysed. Treatment with atorvastatin significantly improved post-MI LV function (fractional shortening, +120%; dP/dt max, +147%; and LV end-diastolic pressure, −27%). Furthermore atorvastatin treatment markedly decreased the levels of TNF-α, IL-6 and MCP-1, reduced myocardial infiltration of macrophages and significantly increased myocardial and serum levels of the anti-inflammatory cytokine IL-10. Thus the balance between pro-inflammatory and anti-inflammatory cytokines was shifted in the anti-inflammatory direction, as shown by a significantly decreased TNF-α/IL-10 ratio. Atorvastatin ameliorated early LV remodelling and improved LV function in rats with HF subsequent to MI. Our study suggests that the modulation of the balance between pro- and anti-inflammatory cytokines towards the anti-inflammatory cytokine IL-10 is one salutary mechanism underlying how atorvastatin influences post-MI remodelling and thus improves LV function.

INTRODUCTION
CHF [chronic HF (heart failure)] is one of the major public health problems in the modern world. Despite major improvements in therapy over the last few years, the mortality rate in patients with CHF still remains at a very high level. Symptomatic HF continues to confer a worse prognosis than the majority of...
cancers in Europe and the United States, with a 1-year mortality averaging 45 % [1]. MI (myocardial infarction) represents one of the most important causes in the development of cardiac failure [2]. Evidence has shown that pro-inflammatory cytokines, such as TNF-α (tumour necrosis factor-α), IL (interleukin)-6 and MCP-1 (monocyte chemoattractant protein-1) are involved in the remodelling process following MI. Furthermore, several studies suggest that the extent of cytokine production directly correlates with the severity of the disease process as well as the mortality rate [3–5]. On the other hand, anti-inflammatory cytokines, such as IL-10, have potent anti-inflammatory properties and may neutralize the effects of pro-inflammatory cytokines [6,7]. In this context, IL-10 has already been identified as a potentially important inhibitory mediator that may be involved in resolution of the post-MI inflammatory reaction [8].

In recent years, accumulating evidence has revealed the anti-inflammatory effects of statins [9–11]. In numerous investigations, it has been shown that administration of statins decreased the levels of CRP (C-reactive protein) and pro-inflammatory cytokines with a concurrent decrease in cardiovascular events even in patients with normocholesterolaemia [12,13]. However, the potential influence of statins on the anti-inflammatory cytokine IL-10 and its effect on cardiac function has not been clearly investigated and is still far from being understood.

Therefore the aim of the present study was to elucidate the effect of atorvastatin on the modulation of the anti-inflammatory cytokine IL-10 and its effect on LV (left ventricular) function in rats with HF subsequent to MI.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats were maintained on standard rat chow and water ad libitum. MI was induced by occlusion of the LAD (left anterior descending) coronary artery. Sham control animals were handled similarly, except the suture around the coronary artery was not tied. Body weight, general behaviour and mortality of the animals were monitored on a regular basis. Cardiac function was assessed 4 weeks after the induction of MI or sham operation.

All animal study protocols were approved by the local authority for Laboratory Animal Care. The study conforms 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).

Induction of MI

MI was induced in adult male Sprague–Dawley rats by ligating the LAD coronary artery according to the technique by Pfeffer et al. [14]. Rats were anaesthetized intraperitoneally with a mixture of xylazine (1 mg/100 g of body weight) and ketamine (10 mg/100 g of body weight), intubated with a fine polyethylene tube and put on positive-pressure ventilation (model 683; Harvard Apparatus). A left intercostal thoracotomy was performed to expose the heart, and the pericardium was opened. The LAD coronary artery was looped by a single nylon suture (5.0) 1 mm from its origin and gently tied. This procedure produced a clearly demarcated (cyanotic and bulged) area of acute ischaemia corresponding to the distribution of the LAD coronary artery distal to the occlusion, which results in MI of the free left ventricle and subsequently HF. After ligation, the heart was repositioned in the chest, the skin was sutured and air in the chest was removed with a syringe. After surgery, all rats were individually caged for a 24-h period of recovery. A sham-surgery group, in which all surgical procedures were the same, except that LAD coronary artery ligation was not performed, was studied and did not present differences when compared with control non-surgically operated rats (results not shown). After this period, all of the rats with MI recovered to good clinical condition and had no respiratory distress or ascites, peripheral oedema or pleural effusion. A mortality rate of approx. 25 % among the animals submitted to LAD coronary artery ligation was observed.

Treatment of animals with atorvastatin

Atorvastatin (Lipitor; Pfizer) was suspended in PBS. On the first day post-MI or sham operation, animals were randomized to receive either placebo (PBS; n = 6) or atorvastatin (10 mg/kg of body weight; n = 6) given by oral gavage for 4 weeks. This dose was chosen because it has been shown to be very effective in rats [15–17].

Echocardiography

Echocardiographic examinations were performed under volatile isoflurane anaesthesia (2.5 % in oxygen; 500–700 ml/min; Draeger and Foehr Medical Instruments). Care was taken to maintain a stable physiological heart rate at approx. 350 beats/min during the experiments as monitored using a three-channel ECG. Chests were shaved and rats were placed in a left lateral decubitus position. For echocardiographic examination, a Vivid 7 ultrasound system (General Electric Healthcare) was used comprising a 10 MHz transducer (S10; General Electric Healthcare). All settings for pre- and post-processing were adapted and optimized for small animals. The penetration depth was 2 cm, near field was focussed and gain was adjusted to optimal delineation. All recordings were stored digitally for subsequent off-line analysis.

Examinations were started in conventional two-dimensional echocardiography with a frame rate of 80/s, resulting in 14 frames/heart cycle. The scan was performed in a parasternal short-axis view to measure LV diameter and endocardial areas in end-diastolic and
Table 1  Haemodynamic and echocardiographic parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham (n = 5)</th>
<th>MI (n = 6)</th>
<th>MI + atorvastatin (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (beats/min)</td>
<td>390 ± 32</td>
<td>410 ± 24</td>
<td>408 ± 31</td>
</tr>
<tr>
<td>Infarct size (%)</td>
<td>—</td>
<td>48.8 ± 3.9</td>
<td>46.3 ± 5.2</td>
</tr>
<tr>
<td>LVSP (mmHg)</td>
<td>129.9 ± 8.5</td>
<td>110.6 ± 7.6*</td>
<td>119.3 ± 8.2</td>
</tr>
<tr>
<td>Mean arterial pressure (mmHg)</td>
<td>101.8 ± 7.3</td>
<td>97.9 ± 6.9</td>
<td>99.8 ± 7.6§</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>5.3 ± 0.8</td>
<td>9.6 ± 0.4†</td>
<td>8.2 ± 0.6</td>
</tr>
<tr>
<td>FS</td>
<td>40 ± 2.24</td>
<td>27.2 ± 4.45§</td>
<td>32.9 ± 3.72†</td>
</tr>
</tbody>
</table>

end-systolic frames, as recommended by the American Society of Echocardiography [18]. The time of end-diastole was therefore defined as the time of maximum diameter of the LV in one heart cycle. Accordingly, end-systole was defined as the minimum diameter. Subsequently, FS (fractional shortening) was calculated.

Haemodynamic measurements
LV pressures were measured via a saline-filled cannula, which was inserted through the right carotid artery and connected to a pressure transducer. The cannula was inserted into the left ventricle to monitor LVSP (LV systolic pressure) and LVEDP (LV end-diastolic pressure), as well as to measure maximum rate of rise of LV pressure (dP/dt).

Sample collection
After completing the cardiac haemodynamic measurements, all the rats had their heart stopped in diastole by an intravenous injection of 10% (w/v) KCl (approx. 2–3 ml). The right ventricles were separated from the heart by the septums. The hearts were immediately frozen in liquid nitrogen and stored at −70 °C.

Myocardial TNF-α and IL-10 protein levels
Hearts were washed with PBS, and viable ventricular tissue was snap frozen in liquid nitrogen. Frozen tissue (0.5–1.0 g) was homogenized, and membrane-bound and soluble fractions of TNF-α and IL-10 proteins were collected and analysed by ELISA using commercially available kits (R & D Systems) [19,20]. Serum TNF-α and IL-10 were measured similarly.

Histological examination
Hearts were stained with haematoxylin or subjected to immunostaining by using antibodies against CD68 (ED-1; Serotec). Immunoreactive materials were visualized by using a streptavidin–biotin staining kit. Macrophages (CD68-positive cells) were counted by a technician blinded to the treatment regimen. As negative controls, immunohistochemistry was performed without the primary antibodies.

Statistical analysis
All results are expressed as means ± S.E.M. For statistical analysis of the data, group means were compared by one-way ANOVA, and a Bonferroni’s test was used to identify differences between groups. Statistical significance was considered to be indicated by a value of P < 0.05. For our sample size and for α 0.05 analysis revealed a power of 0.92.

RESULTS
The mortality in the coronary-artery-ligated groups, during or immediately after the surgery, was approx. 25%. There was no significant difference in body weight gain between the coronary-artery-ligated animals and their respective sham-operated controls. In the atorvastatin-treated MI group, body weight was not different from the non-treated MI group.

Assessment of cardiac function
Echocardiographic measurements and left heart catheterization were performed 4 weeks after surgery (Table 1). In the untreated group, MI resulted in a progressive increase in LV diameter which was slightly, but not significantly, reduced by atorvastatin. LV function in the MI group showed a progressive and significant impairment after MI, as indicated by a decrease in FS that was prevented by treatment with atorvastatin (Table 1).

Inflammatory and anti-inflammatory cytokines in post-MI LV dysfunction
At 4 weeks after coronary artery ligation, levels of myocardial TNF-α and IL-6 increased significantly
Figure 1  Echocardiographic images of rat hearts after sham-operation (A) and induction of MI (B)
Upper panel, two-dimensional echocardiography in the parasternal short-axis plane. Lower panel, representative M-mode short-axis of the left ventricle (LV). ESD, end-systolic diameter; EDD, end-diastolic diameter; RV, right ventricle.

Figure 2 (A) LVEDP and (B) $dP/dt_{\text{max}}$, a parameter of systolic function, in sham-operated rats and rats with MI untreated or treated with atorvastatin for 4 weeks
Results are expressed as means ± S.E.M. *$P < 0.01$ compared with sham-operated rats; †$P < 0.05$ atorvastatin treatment compared with the untreated MI group. atorva, atorvastatin.

(Figures 3B and 3D). Systemically, serum levels of TNF-$\alpha$ (Figure 3C), IL-6 (Figure 3A) and MCP-1 (see Figure 6A) were also found to be significantly increased in the untreated MI group compared with the sham-operated controls. On the other hand, the anti-inflammatory cytokine IL-10 was significantly decreased in its membrane-bound (myocardial) as well as soluble form 4 weeks after MI induction (Figure 4). When comparing the pro-inflammatory with the anti-inflammatory ‘profile’ in the MI group, the TNF-$\alpha$/IL-10 ratio was found to be significantly higher in the MI group (Figure 5).

A 4-week-treatment with atorvastatin significantly decreased myocardial and serum levels of TNF-$\alpha$ as well as IL-6 compared with the untreated MI group (Figure 3). A similar effect was seen for the CC chemokine MCP-1, which was significantly decreased during treatment with atorvastatin (Figure 6A). On the other hand, atorvastatin significantly increased myocardial and serum levels of the anti-inflammatory cytokine IL-10 (Figure 4).

Atorvastatin treatment shifted the balance between pro-inflammatory (TNF-$\alpha$) and anti-inflammatory (IL-10) cytokines towards the anti-inflammatory IL-10, as seen in a significantly decreased TNF-$\alpha$/IL-10 ratio (Figure 5).

Infiltration of macrophages
Macrophages are key regulators of the early LV remodelling process after MI. To determine the rate of macrophage infiltration post-MI, the number of macrophages infiltrated into the infarct zone was quantified (Figure 6B). The myocardium of non-treated MI rats had significantly stronger infiltration of macrophages compared with atorvastatin-treated MI rats (Figure 6B).
Atorvastatin and heart failure

DISCUSSION

The results of the present study show that atorvastatin, when started within 24 h after MI, markedly ameliorated LV systolic function. This effect was accompanied not only by the reduction in myocardial as well as serum levels of pro-inflammatory cytokines (including TNF-α and IL-6) and chemokines (MCP-1), but also by a reduction in myocardial macrophage infiltration. In addition, an increase in the myocardial and serum levels of the anti-inflammatory cytokine IL-10 were also observed.

Several studies have provided evidence that the extent of cytokine production directly correlates with the severity of HF [3,4]. In our present study, we observed a significant increase in myocardial and serum levels of the pro-inflammatory cytokines TNF-α and IL-6 as well as an increase in the CC chemokine MCP-1 at 4 weeks post-MI. On the other hand, the levels of membrane-bound as well as the soluble form of the anti-inflammatory cytokine IL-10 were decreased significantly. Interestingly, this reduction also significantly correlated with depressed cardiac function. As an anti-inflammatory cytokine, IL-10 has already been shown to inhibit the production of pro-inflammatory cytokines (including TNF-α and IL-6), thus suppressing the inflammatory response [21,22]. It has been demonstrated previously that reperfused infarction in IL-10-deficient mice is associated with a 75 % mortality, whereas no death occurred in wild-type animals [23]. Furthermore, a direct correlation between myocardial IL-10 mRNA and HF with time has also been reported [24]. In a previous study, we have shown that patients with advanced CHF [NYHA (New York Heart Association) class III and IV] had significantly decreased levels of serum IL-10 and a significantly increased TNF-α/IL-10 ratio [25].

As over the past decade more and more studies have revealed the anti-inflammatory effects of statins, statins have been tested in experimental settings beyond their well-known cholesterol-lowering properties [26,27]. Bauersachs et al. [28] reported that cerivastatin improved LV remodelling in rats after a large MI, and Tiefenbacher et al. [29] demonstrated that fluvastatin significantly reduced MI size in a reperfusion model. In our present study, in the MI model of permanent coronary artery occlusion, treatment with atorvastatin markedly ameliorated LV remodelling and LV function, supported by an increased FS and dP/dtmax and decreased LVEDP. Furthermore, treatment with atorvastatin not only reduced the levels of TNF-α, IL-6 and MCP-1, but also increased myocardial and serum levels of IL-10, thus shifting the balance between pro-inflammatory and
anti-inflammatory cytokines in the anti-inflammatory direction.

Even though statins have already been shown to up-regulate IL-10 levels in experimental encephalomyelitis [30], rheumatologic therapy [31] and after cardiac surgery [32], their influence on IL-10 and, thus, on post-MI LV function has not been clearly investigated. Our present findings indicate that atorvastatin might improve LV remodelling in part through increasing IL-10 production and inhibiting the expression of TNF-α, IL-6 and MCP-1. Interestingly, animals treated with atorvastatin also had a significant reduction in myocardial infiltration of macrophages 4 weeks post-MI, suggesting further its role in reducing inflammation and thus influencing cardiac function. One possible explanation for the reduced macrophage infiltration could be the decrease in the levels of the CC chemokine MCP-1; however, the exact molecular mechanisms underlying the differential regulation of pro- and anti-inflammatory cytokines by atorvastatin are far from clear. Ortego et al. [33] reported that atorvastatin reduced NF-κB (nuclear factor κB) activity, which preferentially promotes pro-inflammatory cytokine expression. In another report [34], our group has show that statins down-regulate CD40 expression, another important member of the TNF-receptor family with potent influence in chronic inflammatory disease states. On the other hand, up-regulation of IL-10 by atorvastatin may be a potent mechanism in further down-regulating pro-inflammatory cytokines (including TNF-α and...
IL-6) and chemokines (MCP-1) [35–37]. Even though there are many reports that atorvastatin can markedly decrease the production of pro-inflammatory cytokines and chemokines [10,11], our present study shows its effect on regulating the inflammatory balance towards the anti-inflammatory cytokine IL-10 and thus influencing post-MI LV remodelling and function in an animal model.

Although there are clinical reports that confirm the beneficial effects of these experimental studies [38,39], the recently published CORONA study (Controlled Rosuvatin in Multinational Trial in Heart Failure), one of the first large randomized explorations of a statin given explicitly for HF, did not show a significant effect on cardiovascular outcomes [40]. However, that study showed a significantly reduced number of hospitalizations from cardiovascular causes and from HF. In our present study, we observed a significant influence of atorvastatin on cardiac function when starting the therapy within 24 h post-MI, suggesting that giving statins earlier in the natural history of HF might produce better results. As there are reports that patients with low IL-10 levels might have a poorer cardiovascular prognosis [41,42], it is tempting to hypothesize that those patients in particular could benefit from a therapy that shifts the inflammatory balance in an anti-inflammatory direction. However, further studies need to be done to shed light on some of these unresolved questions.

In summary, our present study indicates that pro- and anti-inflammatory cytokines are important players in post-MI LV remodelling. The effect of atorvastatin on the modulation of the balance between pro- and anti-inflammatory cytokines towards the anti-inflammatory cytokine IL-10 is one salutary mechanism of how atorvastatin influences post-MI remodelling and thus improves post-MI LV function. These findings support further the concept that the balance between pro- and anti-inflammatory cytokines is a major determinant in the prognosis of post-MI HF and, therefore, enhancing anti-inflammatory cytokines may be a promising approach for medical treatment.

ACKNOWLEDGMENTS

The study was supported by a grant of the IZKF (Interdisciplinary Center for Clinical Research) within the Medical Faculty of the University of Erlangen, Erlangen, Germany.

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