Basic Res Cardiol 96: 345 – 352 (2001)

ORIGINAL CONTRIBUTION

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Monocyte chemoattractant protein-1 enhances and interleukin-10 suppresses the production of inflammatory cytokines in adult rat cardiomyocytes

Received: 10 October 2000
Returned for 1. revision: 2 November 2000
1. Revision received: 5 December 2000
Returned for 2. revision: 2 January 2001
2. Revision received: 5 January 2001
Accepted: 8 January 2001

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Abstract *Objective* Chemokines control the migration of leukocytes to inflamed tissue, and in particular monocyte chemoattractant protein (MCP)-1 has been implicated in the pathogenesis of several cardiovascular disorders such as chronic heart failure (CHF) and myocarditis. We hypothesised that MCP-1 may directly contribute to an inflammatory response in the cardiomyocytes, and in the present study we examined in adult rat cardiomyocytes: (*i*) the effect of tumour necrosis factor (TNF)α on MCP-1 production, (*ii*) the effect of MCP-1 on production of other inflammatory cytokines, and (*iii*) if the anti-inflammatory cytokine interleukin (IL)-10 could suppress any TNFα-induced MCP-1 production. *Methods* We used enzyme immunoassays, RNase protection assays and slot blot analysis to measure protein and mRNA levels of various cytokines in adult rat cardiomyocyte cultures. *Results* (*i*) We found a ~6.4-fold increase of the MCP-1 level accompanied by an increase in MCP-1 mRNA accumulation in cardiomyocyte cultures after TNFα stimulation. (*ii*) In contrast, TNFα had no effect on IL-10 and only a modest effect on IL-1β and IL-6 levels in these cells. (*iii*) Importantly, MCP-1 stimulated inflammatory response in cardiomyocytes by enhancing IL-1β and IL-6 levels in these cells as found at both the protein and mRNA level. (*iv*) Co-stimulation with IL-10 resulted in a ~55 % reduction in TNFα-stimulated MCP-1 levels in cardiomyocyte culture supernatants. *Conclusion* The present study demonstrates for the first time that MCP-1 can directly affect cardiomyocytes, and we introduce MCP-1 as a potential enhancer and IL-10 as a potential suppresser of inflammatory responses within the myocardium.

Key words Tumour necrosis factor α – monocyte chemoattractant protein-1 – interleukin-10 – gene expression – protein

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Introduction

The attraction of leukocytes to tissue is essential for inflammation and the host response to infection. This process is partly controlled by chemokines, which are chemotactic cytokines (1, 2). Recently, chemokines have been implicated in the pathogenesis of several inflammatory disorders such as asthma and rheumatoid arthritis (3, 4), and there is also growing evidence suggesting that these cytokines may play a pathogenic role in several cardiovascular disorders (5). The involvement of chemokines in the pathogenesis of atherosclerosis has been widely investigated, and it seems that monocyte recruitment to the vessel wall is mainly regulated by chemokines, particularly monocyte chemoattractant protein (MCP)-1 (6). MCP-1 has also been implicated in the pathogenesis of myocarditis, acute myocardial infarction and ischemia-induced myocardial damage (7–9), and we and others have recently suggested a role for MCP-1 in the development and progression of chronic heart failure (CHF) (10–12).

MCP-1 may be produced in various cell types, but at present data on MCP-1 production in the myocardium are scarce. Moreover, there is no evidence for a direct effect of MCP-1 on cardiomyocytes. We hypothesised that MCP-1 may contribute to an inflammatory response within the myocardium and in the present study this hypothesis was examined by different experimental approaches. First, we examined if tumour necrosis factor (TNF)α, a cytokine that has been shown to directly induce dysfunction of cardiomyocytes, could induce MCP-1 production in adult rat cardiomyocytes. The MCP-1-inducing effect of TNFα was compared with the TNFα stimulated production of interleukin (IL)-6 and IL-1β in these cells, two inflammatory cytokines with direct effects on cardiomyocytes (e.g. impairment of contractility and induction of cardiac hypertrophy) (13–15). Second, we examined if MCP-1 may further enhance an inflammatory response within the myocardium by directly stimulating cytokine production in cardiomyocytes. Finally, there is growing evidence suggesting that the anti-inflammatory cytokine IL-10 is involved in modulation of inflammatory disorders (16, 17), and one recent report suggest that IL-10 may have beneficial effects on inflammatory processes within the myocardium (18). However, it is not known if IL-10 inhibits the inflammatory response at the level of the cardiomyocyte. We therefore also investigated whether IL-10 could down-regulate any TNFα induced MCP-1 production in adult cardiomyocyte cultures.

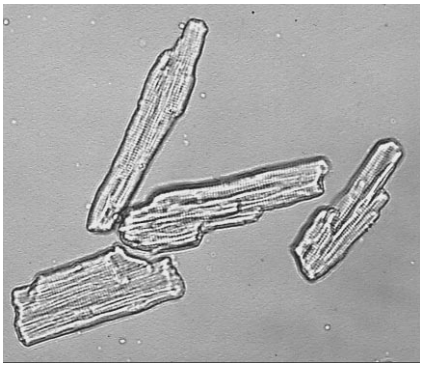


Fig. 1 Representative photograph of quiescent ventricular cardiomyocytes.

Methods

Isolation of adult rat cardiomyocytes

Myocytes were isolated from the ventricles of male Wistar rats (250 – 300 g) as described (19) (Fig. 1). Following pentobarbital anaesthesia, hearts were removed and subsequently perfused for 5 minutes via aorta in a modified Langendorff system with a modified minimum essential medium [Joklik MEM (Gibco, 072-2300A), 23.8 mM $NaHCO_3$ (Merck), 1 mM $MgSO_4 \cdot 6\,H_2O$ (Merck), 1 mM DL-Carnitine (Sigma) and 1000 mL deionised H_2O equilibrated with 5 % CO_2 – 95 % O_2 (37 °C, pH 7.4)]. Thereafter, the hearts were perfused for 25 minutes with the modified minimum essential medium added with 200 U/mL collagenase (Worthington, 4176, Freeland, NJ) and 0.1 % bovine serum albumin (BSA, Sigma). Subsequently, the atria and great vessels were removed, the ventricular tissue minced and gently shaken for 15 minutes at 37 °C in modified minimum essential medium added with 0.1 % BSA, 200 U/mL collagenase and 0.02 mg/mL DNase (Worthington, 2006). The cell solution was centrifuged for one minute at 400 rpm, the supernatant removed, and the cells resuspended in 15 mL modified minimum essential medium with 0.25 mM $CaCl_2$ and 1 % BSA. Then centrifugation was repeated, the supernatant removed and the cell solution resuspended in 15 mL modified minimal essential medium with 0.5 mM $CaCl_2$ and 1 % BSA. The cell solution was filtered through a nylon filter (GyroMed, 200 μm) and allowed to sediment before the supernatant was removed. Then the cell sediment was carefully layered on top of 20 mL modified minimum essential medium containing 6 % BSA and the cells were allowed to sediment for 10 minutes. Cardiomyocytes settle faster than other cell types which remain in the upper layer of the solution. The upper layer was then removed. This procedure was repeated one more time, and provided us with a pure cell preparation of rod shaped cardiomyocytes. We examined the cell solution before plating, and endothelial cells and blood cells could not be observed in a light microscope. After 48 hours in culture less than 4 % of the cells

were fibroblasts. Cells were cultured on natural mouse laminin (1 μg/mL, Gibco 23017-015) in MEDIUM 199 (M7528, Sigma) mixed with 2 mg/mL BSA, 2 mM DL-carnitine, 5 mM creatine, 5 mM taurine, 0.1 μM insulin, 10^{-10} M triiodothyronine (all from Sigma), 100 U/mL penicillin (Gibco) and 100 μg/mL streptomycin (Gibco) equilibrated with 5 % CO_2 – 26 % O_2 (37 °C, pH 7.4). The investigation 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).

Cytokine production assay

Two hours after primary cardiomyocyte culture rat TNFα (10 – 60 ng/mL), rat MCP-1 (10 – 100 ng/mL) (both from R&D Systems, Minneapolis, MN) and rat IL-10 (5 ng/mL) (Biosource, Camarillo, CA) were added to cell cultures alone or in different combinations as indicated in Figs. 2, 4 and 6. After 24 and 48 hours of culture (37 °C, pH adjusted to 7.4) cell-free supernatants and cardiomyocytes were harvested and stored at –80 °C. Endotoxin levels were tested in all media, buffers and stimulants used in the study and were < 10 pg/mL (Limulus amebocyte test).

Enzyme immunoassay (EIA)

IL-1β, IL-6, MCP-1 and IL-10 in cell culture supernatants were measured by EIAs (all from Biosource) for detection of rat cytokines according to the manufacturer's descriptions. In our laboratory, the intra- and interassay coefficients of variation were < 9 % for all EIAs.

mRNA analysis

Following isolation, ventricular rat myocytes (~1 mill) were cultured in 162 cm^2 tissue culture flasks (Costar, New York, NY) coated with laminin (10 μg/ml) from which the disposable cover had been removed in a sterile hood as described by Holt et al. (20). mRNA was extracted from about 10^6 cultured cardiomyocytes using oligo(dT)-conjugated paramagnetic beads (Dynal A/S, Oslo, Norway), according to the manufacturer's instruction.

Slot blot analysis was used for the detection and quantification of rat MCP-1 mRNA levels. A slot blot manifold (Minifold II, Schleicher & Schuell GmbH, Dassel, Germany) was used to apply the mRNA samples (~1 μg) on a nylon membrane. Filters were prehybridised for 3 hours followed by hybridisation to ^{32}P-labelled MCP-1 rat cDNA probe overnight at 42 °C, and washing in 2 x SSC/0.1 % SDS for 5 x 5 minutes at room temperature and then in 0.1 x SSC/0.1 % SDS for 2 x 15 minutes at 60 °C. The mRNA signal was quantified using phosphorimaging (Fujifilm, Elmsford, NY) and normalised to the signal obtained by hybridisation with an end labelled oligo(dt)18S probe (Eurogentec, Seraing, Belgium). Northern blot analysis of rat mRNA with the MCP-1 cDNA probe was performed prior to slot blot analysis to test the specificity of the probe.

RNase protection assays (RPA) were used for the detection and quantification of rat IL-1β and IL-6 mRNA levels. Multi-probe (rCK-1) was available with reagents for *in vitro* transcription and RPA (RiboQuant; Pharmingen, San Diego, CA). The anti-sense RNA probes of IL-1β, IL-6 and the housekeeping gene glyceraldehyd-3-phosphate dehydrogenase (GAPDH) able to hybridise with target human mRNA were synthesised.

For all hybridisation assays, ~0.5 μg mRNA was mixed with 0.5 – 2x10^6 cpm probe (α-^{32}P-UTP, 3000 Ci/mmol). Protected fragments were separated in a denaturing 6 % polyacrylamide gel for 90 minutes. The dried gel was exposed to a phosphorimaging screen (Cyclone system; Packard, Meriden, CT) for 20 hours followed by densitometric analysis using 1D Quantifier (Phoretix, Newcastle, England). The mRNA signal was normalised to the signal obtained from GAPDH mRNA.

Statistics

Values are expressed as means ± SE. For comparison of two groups, the Mann-Whitney U test (two tailed) was used. When more than two groups were compared, one-way ANOVA and the Bonferoni multiple comparisons procedure were employed. Values of $P < 0.05$ were considered significant.

Results

The effect of TNFα stimulation on IL-1β, IL-6, IL-10 and MCP-1 production in isolated adult rat cardiomyocytes

We first examined whether TNFα could induce MCP-1 production in adult rat cardiomyocytes by measuring MCP-1 level in cardiomyocyte cultures exposed to various concentration of TNFα (10 – 60 ng/mL) for 24 and 48 hours. For comparison, we also measured the effect of TNFα on IL-1β, IL-6 and IL-10 levels. Compared to levels found in unstimulated cultures, TNFα induced an ~6.4-fold increase in MCP-1 levels in a dose-dependent manner (Fig. 2A). Time kinetics showed that MCP-1 was detected after 24 hours, and the maximum effect was observed 48 hours after TNFα stimulation (data not

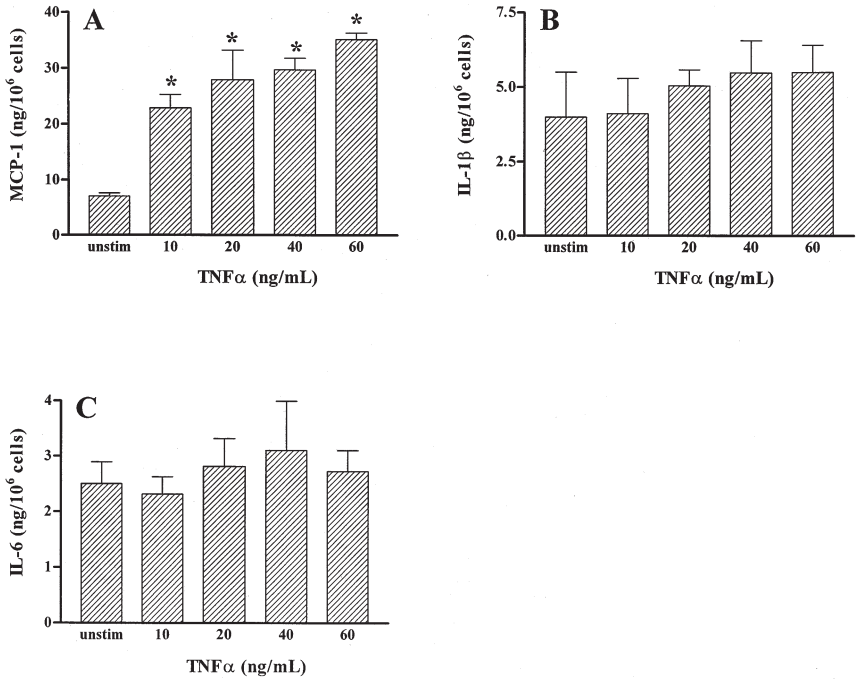


Fig. 2 Dose effect of TNFα on MCP-1 (**A**), IL-1β (**B**) and IL-6 (**C**) levels (ng/10^6 cells) in rat cardiomyocyte supernatants after 48 (MCP-1) and 24 hours (IL-1β and IL-6). The mean MCP-1 level after TNFα stimulation was 28.7 ± 3.2 ng/10^6 cells or 2.7 ± 0.4 ng/ml. Data are given as means of 5 separate experiments (±SE). *$P < 0.05$ vs. unstimulated cells. # $P < 0.05$ vs. TNFα 10 ng/ml.

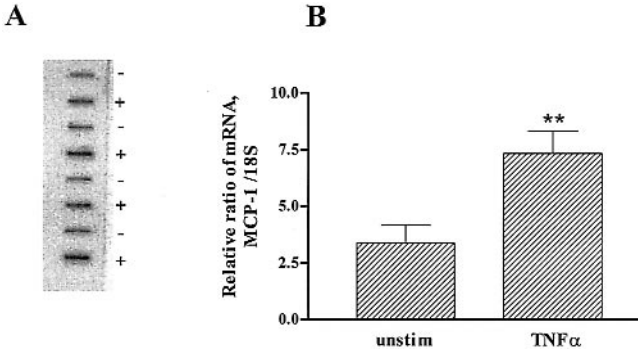
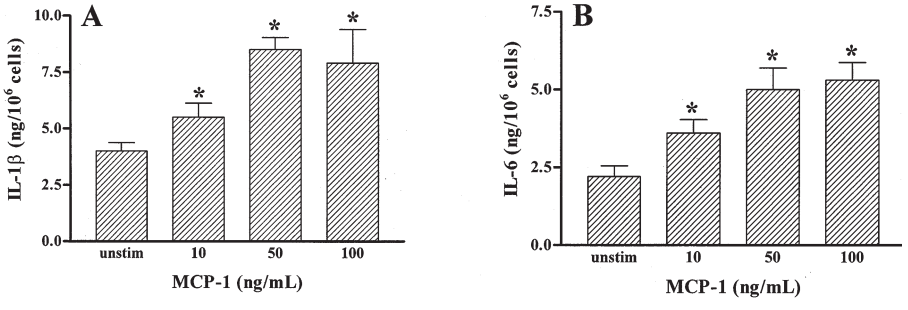


Fig. 3 Representative autoradiographic image (**A**) and bar graphs (**B**) of slot blot analysis showing levels of MCP-1 mRNA in unstimulated (–) and TNFα-stimulated (40 ng/mL) (+) rat cardiomyocytes (10^6 cells) after 48 hours in 6 separate experiments (±SE). **$P < 0.01$ vs. unstimulated cells.

shown). In contrast, TNFα induced only a modest increase in IL-1β (~1.3-fold) and IL-6 levels (~1.2-fold), reaching maximum levels after 24 hours (Fig. 2B–C). As for the anti-inflammatory cytokine IL-10, no detectable levels were found in either unstimulated or TNFα stimulated cell cultures (data not shown).

We also investigated whether the increased MCP-1 level after TNFα stimulation was accompanied by increased MCP-1 mRNA accumulation. As shown in Fig. 3, the level of MCP-1 mRNA was low in unstimulated cardiomyocytes, but its expression was significantly stimulated (~2.2-fold increase) by exposure to TNFα (40 ng/mL) for 48 hours.

Fig. 4 Dose effect of MCP-1 on IL-1β (**A**) and IL-6 (**B**) levels (ng/10^6 cells) in rat cardiomyocyte supernatants after 24 (IL-6) and 48 (IL-1β) hours. Data are given as means of 5 separate experiments (±SE). *$P < 0.05$ vs. unstimulated cells.



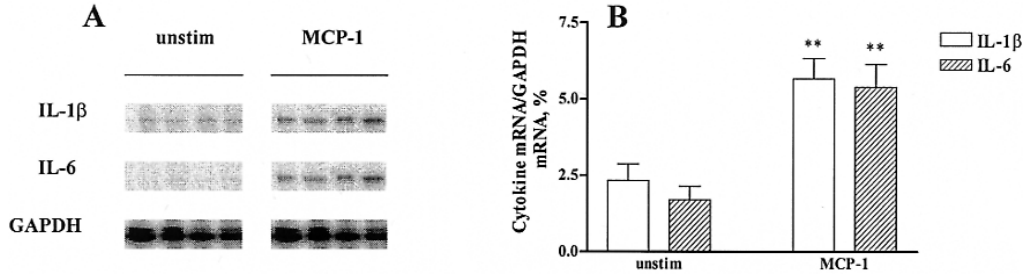


Fig. 5 Representative autoradiographic image (**A**) and bar graphs (**B**) of RNase protection assays showing levels of IL-1β and IL-6 mRNA in relation to GAPDH mRNA in unstimulated and MCP-1 stimulated (100 ng/mL) rat cardiomyocytes (10^6 cells) after 48 hours in 8 separate experiments (±SE). $^{**}P < 0.01$ vs. unstimulated cells.

The effect of MCP-1 on IL-1β and IL-6 production in isolated adult rat cardiomyocytes

Since MCP-1 is produced in adult rat cardiomyocytes during TNFα stimulation, we next investigated whether MCP-1 may further enhance the inflammatory response in these cells by measuring levels of IL-1β and IL-6 in cardiomyocyte cultures exposed to various concentrations of MCP-1 (10 – 100 ng/mL) for 24 and 48 hours. Compared to levels found in unstimulated cell cultures, MCP-1 induced an increase in both IL-1β and IL-6 levels (~2.1-fold and ~2.4-fold, respectively) in a dose-dependent manner (Fig. 4). Time kinetics showed that the maximum IL-1β and IL-6 levels were observed 48 and 24 hours after MCP-1 stimulation, respectively (Fig. 4). No effect of MCP-1 was found on TNFα and IL-10 levels (data not shown).

We then investigated whether the increased IL-1β and IL-6 levels after MCP-1 stimulation of cardiomyocytes were accompanied by increased IL-1β and IL-6 mRNA accumulation in these cells. As shown in Fig. 5, the levels of IL-1β and IL-6 mRNA were low in unstimulated cardiomyocytes, but the expression of these cytokines were significantly enhanced (~2.4-fold and ~3.2-fold, respectively) by exposure to MCP-1 (100 ng/mL) for 48 hours.

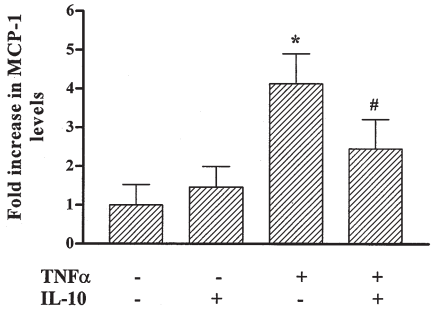


Fig. 6 Effect of IL-10 (5 ng/mL) on TNFα (40 ng/mL) induced MCP-1 levels (ng/10^6 cells) in rat cardiomyocyte supernatants after 48 hours in 5 separate experiments (±SE). $^{*}P < 0.05$ vs. unstimulated cells. # $P<0.05$ vs. TNFα only.

The effect of IL-10 on TNFα-induced MCP-1 production in adult rat cardiomyocytes

Although we could not detect any IL-10 protein in either unstimulated or TNFα stimulated cardiomyocytes cultures, IL-10 might *in vivo* reach the myocardium by infiltrating monocytes and T cells (16, 17). To investigate whether the stimulatory effect of TNFα could be modulated by IL-10, we examined the effect of IL-10 (5 ng/mL) on TNFα (40 ng/mL) induced MCP-1 response after 48 hours of culture. While IL-10 did not influence the MCP-1 level when given alone, IL-10 resulted in a marked reduction (~55 %) in the TNFα-stimulated MCP-1 protein level in cardiomyocyte culture supernatants (Fig. 6). Similar patterns were found whether IL-10 was added to cell culture 2 hours before or simultaneously with TNFα stimulation (data not shown).

Discussion

The present study demonstrates that MCP-1 production is markedly enhanced by TNFα in adult rat cardiomyocytes as confirmed at both the mRNA and protein level. Moreover, we show that MCP-1 can further modulate cytokine production within the myocardium by significantly increasing both IL-1β and IL-6 production in cardiomyocytes. Finally, MCP-1 production induced by TNFα was markedly suppressed when the cardiomyocytes were co-stimulated with IL-10. Inflammatory cytokines have recently attracted considerable attention as an important pathogenic factor in the development and progression of CHF (13–15, 21–23). The present study for the first time demonstrates that MCP-1 can directly affect cardiomyocytes, and we introduce MCP-1 as a potential enhancer and IL-10 as a potential suppresser of inflammatory responses within the myocardium.

Our findings suggest that cardiomyocytes have an active role in myocardial inflammatory processes, not

only by responding to cytokines, but also by producing several cytokines spontaneously and after stimulation with other cytokines. Our findings in the present study suggest that MCP-1 is an important mediator of this inflammatory response within the myocardium. Thus, we confirm and extend previous reports (24, 25) showing that while TNFα has no effect on IL-10 and only modest effects on IL-1β and IL-6 levels in adult rat cardiomyocytes, this cytokine is a potent inducer of MCP-1 in these cells. Even more importantly, we show for the first time that MCP-1 can directly stimulate cardiomyocytes by demonstrating an enhancing effect of MCP-1 on IL-1β and IL-6 production in these cells.

Chronic low grade inflammation as reflected in activated vascular endothelium and the presence of infiltrating inflammatory cells have been found in the failing myocardium (26, 27), and we have hypothesised that MCP-1 and other chemokines may be important mediators in this process. Indeed, MCP-1 has been found to induce monocyte infiltration in murine cardiac muscle with development of a number of pathological changes characteristic of CHF, e.g. cardiac hypertrophy, ventricular dilatation and depressed contractile function (7). Our findings in the present study suggest that the cardiomyocytes themselves may directly contribute to such an infiltration of inflammatory cells within the myocardium.

Besides chemotaxis and leukocyte activation, MCP-1 and other chemokines may also regulate several other biological processes with importance to the pathogenesis of heart failure, e.g. fibrosis, angiogenesis and apoptosis (28–30). Notably, we have recently shown increased gene expression of the MCP-1 receptor (CCR2) in the failing human myocardium, indicating that MCP-1 may directly play a role in the pathogenesis of CHF (11). In the present study, we show that MCP-1 stimulation of cardiomyocytes may induce other inflammatory cytokines, i.e. IL-1β and IL-6 which in turn may reduce cardiomyocyte contractility and increase cardiac hypertrophy (13–15). Furthermore, both IL-1β and IL-6 may induce MCP-1 production in several cell types (31), and if such an effect also exists within the myocardium, it may represent a vicious inflammatory circle, possibly involved in the development of myocardial failure if inappropriately activated.

In our experiments we used a higher MCP-1 concentration (10 – 100 ng/mL) than produced from cultured cardiomyocytes (2.7 ± 0.4 ng/mL). However, we observed a significant increase in both IL-6 and IL-1β production after stimulation with 10 ng/mL MCP-1, suggesting a potential stimulatory effect even at lower MCP-1 concentrations. We feel that the concentrations used in the *in vitro* experiments (10 – 100 ng/mL) are as relevant to the *in vivo* situation as those produced in TNFα stimulated cardiomyocyte cultures in vitro (~3 ng/mL). We believe that the MCP-1 concentration in myocardial tissue, which is exposed to various stimuli from for example leukocytes and fibroblasts, may be higher than the MCP-1 levels in our *in vitro* system for isolated cardiomyocytes where MCP-1 will be diluted in culture medium.

A major finding in the present study was the markedly suppressing effect of IL-10 on TNFα-induced MCP-1 release from adult rat cardiomyocytes. IL-10 is primarily produced by monocytes and T cells and seems to play an important role in controlling cell-mediated and inflammatory responses (16, 17). Indeed, IL-10 deficient mice are characterised by increased leukocyte recruitment upon exposure to lipopolysaccaride (32), and IL-10 has been found to suppress production of reactive oxygen species, TNFα and chemokines from macrophages and lymphocytes (33, 34). Here we show for the first time that IL-10 also may suppress inflammatory responses in cardiomyocytes by reducing TNFα stimulated release of MCP-1. The mechanisms for this effect of IL-10 are uncertain, but may involve downregulation or shedding of TNF receptors in cardiomyocytes or interaction at the intracellular level (35). Whatever the mechanisms, our findings suggest that IL-10 has a potential to counteract the TNFα induced inflammatory responses in the myocardium. Interestingly, IL-10 was recently found to attenuate myocardial lesions in experimental viral myocarditis, possibly by suppressing TNFα production in infiltrating monocytes and lymphocytes in the heart (18). Such a combined suppressive effect on both TNFα production in leukocytes and TNFα mediated effects in cardiomyocytes, as shown in the present study, clearly illustrates the potential anti-inflammatory properties of this cytokine.

The present study shows that MCP-1 is induced by TNFα in adult rat cardiomyocytes and that MCP-1 may further enhance the inflammatory response within the myocardium by inducing IL-1β and IL-6 production in these cells. In contrast to this MCP-1 mediated enhancement of the inflammatory response, IL-10 markedly suppressed the TNFα stimulatory effect on MCP-1 production by a direct effect on the cardiomyocytes. Our findings support a role for MCP-1 as an important local mediator of the inflammatory response within the myocardium. Moreover, suppression of MCP-1 production by IL-10 at the level of the cardiomyocytes suggests that IL-10 has a potential for specific inhibition of inflammatory cytokines within the myocardium.

Acknowledgments This work was supported by the Norwegian Council of Cardiovascular Disease, Research Council of Norway, Medinnova Foundation, Johan H. Andresen's Medical Foundation, Alf and Aagot Helgesens's Legacy and Anders Jahre's Fund for the promotion of Science.

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