Monocyte Chemoattractant Protein-1 is Upregulated in Rats With Volume-Overload Congestive Heart Failure

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Background—Chemokines are potent proinflammatory and immune modulators. Increased expression of chemokines, eg, monocyte chemoattractant protein-1 (MCP-1), has recently been described in clinical and experimental heart failure. The present report is aimed at exploring the expression, localization, and binding site regulation of MCP-1, a member of the C-C chemokine family, in a rat model of volume-overload congestive heart failure (CHF).

Methods and Results—An aortocaval fistula was surgically created between the abdominal aorta and inferior vena cava. Rats with CHF were further subdivided into compensated and decompensated subgroups. Northern blot analysis and real-time quantitative polymerase chain reaction demonstrated upregulation of MCP-1 mRNA expression correlating with the severity of CHF (288±22, 502±62, and 826±138 copies/ng total RNA for sham, compensated, and decompensated animals, respectively; n=5, P<0.05). MCP-1 protein was localized by immunohistochemistry in cardiomyocytes, vascular endothelium and smooth muscle cells, infiltrating leukocytes, and interstitial fibroblasts, and its intensity increased with severity of CHF. In addition, rats with CHF displayed a significant decrease of 125I-labeled MCP-1 binding sites to myocardium-derived membranes (384.3±57.0, 181.3±8.8, and 123.3±14.1 fmol/mg protein for sham, compensated, and decompensated animals, respectively).

Conclusions—Volume-overload CHF in rats is associated with alterations in the expression, immunohistochemical localization, and receptor binding of the MCP-1 chemokine in the myocardium. These changes were more pronounced in rats with decompensated CHF. The data suggest that activation of the MCP-1 system may contribute to the progressive cardiac decompensation and development of CHF in rats with aortocaval fistula. (Circulation. 2000;102:1315-1322.)

Key Words: cardiomyopathy • immunohistochemistry • molecular biology • receptors

Proinflammatory cytokines have been implicated in clinical deterioration in congestive heart failure (CHF) since an increase in plasma as well as myocardial levels of the cytokine tumor necrosis factor (TNF-α) was found in patients with advanced heart failure due to ischemic or idiopathic cardiomyopathies.1–4 TNF-α and other proinflammatory cytokines, such as interleukin (IL)-1β and IL-6 or interferon-γ are also known to induce several chemotactic polypeptides known as chemokines.5,6 The locally acting chemokines, which have the primary function of inducing chemotaxis, constitute a family of >40 known polypeptides, classified into subfamilies on the basis of the relative position of their cysteine residues.7

Serological studies have shown upregulation of certain chemokines in human CHF.8 In particular, monocyte chemoattractant protein (MCP)-1 is a C-C chemokine produced in a variety of cells in response to injury or exposure to other cytokines, such as IL-1, IL-6, TNF-α, or interferon-γ.6,9 The information about MCP-1 involvement in experimental heart failure is scant and incomplete. Transgenic overexpression of MCP-1 in the myocardium resulted in myocarditis and subsequent development of heart failure.10 Pressure overload–induced heart failure in hypertensive animals led to induction of MCP-1.11 To date, the contribution of MCP-1 to the syndrome of heart failure is attributed solely to its potent chemoattractant properties on monocytes, the presence of which has been documented in cardiac specimens of heart failure. Interestingly, angiotensin II (Ang II), which plays a dominant role in the pathogenesis of CHF, also induces MCP-1 gene expression in vivo and in vitro.12,13

Rats with aortocaval fistula (ACF) develop volume-overload–induced cardiomyopathy characterized by marked cardiac dilatation and hypertrophy and subsequently CHF.14–16 This experimental model of CHF is characterized by renal,
hemodynamic, and neurohormonal alterations that closely mimic the changes observed in patients with severe heart failure.\(^{14-17}\) Furthermore, rats with ACF can be subdivided, on the basis of their daily urinary sodium excretion, into compensated and decompensated subgroups, which differ in the degree of severity of CHF.\(^{14}\) Thus, in addition to renal retention of sodium and water, rats with decompensated CHF display a marked degree of neurohormonal activation compared with the compensated subgroup.\(^{14,15}\)

Therefore, in the present study, we characterized the changes in the expression of MCP-1 mRNA and protein in rats with compensated and decompensated heart failure, using a novel real-time polymerase chain reaction (PCR) technique\(^{18}\) combined with standard Northern blotting and immunohistochemistry. In addition, because no data are available on MCP-1 receptors in the myocardium in CHF, binding assays were performed to confirm the presence of and characterize the alterations in MCP-1 receptor in this experimental model of heart failure.

**Methods**

Studies were performed on male Wistar rats weighing \(\approx 300\) g. Animals were kept in temperature- and light-controlled rooms. Tap water and standard rat chow were provided ad libitum. Experiments were conducted in accordance with the **Guide for the Care and Use of Laboratory Animals** (US Department of Health, NIH publication 85-23).

**Experimental Model**

An abdominal ACF was surgically created according to the method of Stumpe et al.\(^{19}\) Briefly, rats were anesthetized with pentobarbital sodium (40 mg/kg). The vena cava and aorta were exposed via a midline abdominal incision. A side-to-side (1.0- to 1.2-mm) anastomosis was created distal to the origin of the renal arteries. Rats were allowed to recover and placed in individual metabolic cages for daily urine collection. The urine was analyzed for UNaV with a Beckman Liquiflo fluorospectrophotometer. Systolic blood pressure was measured by a noninvasive tail-cuff method with an ITTC Life Science Apollo Analyzer, model 179BP. For tissue collection, rats were anesthetized with pentobarbital sodium 24 hours, 3 days, and 7 days after surgery. Their hearts were removed, immediately frozen in liquid nitrogen, and stored at \(-80^\circ\)C.

**Echocardiography**

Animals were anesthetized with isoflurane (1% to 1.5%). Echocardiographic images were obtained with an ATL HDI5000 ultrasound unit with an L12-5 linear array transducer. 2D guided M-mode recordings of the left ventricle in the short-axis view at the level of the papillary muscles were obtained, and left ventricular dimensions and wall thicknesses were measured.

**RNA Extraction**

Total RNA was extracted from cardiac tissue from both ventricles as described by Chomczynski and Sacchi\(^{20}\) after homogenization in a commercial solution (Trizol, Gibco) and quantified by spectrophotometry at 260/280 nm.

**Northern Blot Analysis**

Standard Northern blotting was used to investigate the time course of MCP-1 expression as described in detail previously.\(^{21}\) Total RNA (25 \(\mu\)g) was size-fractionated by electrophoresis on 1.0% agarose gels and transferred onto a GeneScreen Plus membrane (DuPont-New England Nuclear). Ribosomal protein L32 (rpL32) was used as a housekeeping gene.\(^{21}\) Primers were designed according to published sequences (Table 1). Probes were obtained by PCR, gel-purified, labeled with [\(\alpha\)-\(\text{32P}\)]dATP (3000 Ci/mmol; Amersham Corp) by a random-priming DNA labeling kit (Boehringer Mannheim), and hybridized at 42°C overnight.

**TaqMan Real-Time Reverse Transcription–PCR Analysis**

The principle of real-time PCR detection is based on the fluorogenic 5’ nuclelease assay.\(^{18}\) The TaqMan probes were labeled with a 5’ reporter dye, 6-carboxyfluorescein, and a 3’ quencher dye, 6-carboxy-tetramethyl-rhodamine. During the PCR reaction, the AmpliTaq Gold DNA polymerase cleaves the TaqMan probe at the 5’ end and separates the reporter dye from the quencher dye only if the probe hybridizes to the target. This cleavage results in the fluorescent signal generated by the cleaved reporter dye and is directly monitored by the ABI Prism 7700 Detection System.
(Perkin-Elmer). The increase in the fluorescence signal is proportional to the amount of specific PCR product.18

The PCR primers and TaqMan probes were designed with a software program from Perkin-Elmer (Table 1). Specificity test, reverse transcription, and PCR were carried out as described.22 Absolute copy numbers of the target transcripts per nanogram of transcribed total RNA were determined with cloned plasmid DNA for rpl32 and MCP-1 as described earlier.22 The MCP-1 clone was generated by use of the PCR product of the TaqMan sense primer and the Northern antisense primer (Table 1). Data were analyzed with a Sequence Detector V1.6 program (Perkin-Elmer).

Immunohistochemistry

Frozen sections (6 μm thick) were cut and air dried for 1 hour. Sections were fixed in acetone for 10 minutes at −20°C and blocked with 1% FBS (Sigma Chemical Co). Antibodies against rat MCP-1 (Santa Cruz), factor VIII (Sigma), and ED-1 (macrophage marker; Sigma) were applied for 1 hour, appropriately diluted. After a wash in PBS and incubation with peroxidase-conjugated secondary antibody for 45 minutes followed by another wash in PBS, sections were developed in diaminobenzidine substrate (Vector), counterstained with hematoxylin, and coverslipped with Cytoseal (Stephens Scientific). As control, 1 hour preincubations of MCP-1 antibody and peptide (Santa Cruz) and experiments omitting the primary antibody were done.

MCP-1 Binding Studies

Membranes were prepared from frozen heart tissues isolated by differential centrifugation from 3 rats from each group (control, compensated, and decompensated CHF). Briefly, tissues were homogenized in 10 volumes of ice-cold buffer containing 10 mmol/L Tris-HCl (pH 7.4), 5 mmol/L EDTA, 0.1 mmol/L PMSF, 0.1 mg/mL of bacitracin, and 0.1 mmol/L aprotinin. The homogenates were centrifuged at 1000g for 10 minutes at 4°C, and the pellets were discarded. The supernatants were centrifuged at 37,000g for 20 minutes at 4°C; the membrane pellets were washed twice, and the protein content was finally adjusted to 2 mg/mL in incubation buffer.

Table 2 presents selected cardiovascular and renal parameters of control and experimental groups. Rats with decompensated CHF were characterized by a significantly lower UNaV, bradycardia, and a lower systolic blood pressure compared with either compensated or control animals. Also, the heart-to-body weight ratio was significantly higher in rats with decompensated CHF. Similar findings have been reported previously.15 Echocardiography demonstrated that decompensated CHF was associated with a marked degree of cardiac dilatation (Table 2).

MCP-1 Expression: Northern Blot Analysis

Figure 1 presents the data on the expression of MCP-1 and rpl32 mRNA, by Northern hybridization, in control and ACF animals at 24 hours, 3 days, and 7 days, respectively. The data on day 7 in the ACF animals are further subdivided into compensated and decompensated CHF. An increased expression of MCP-1 was observed from day 1 in ACF animals compared with sham-operated animals. MCP-1 mRNA in decompensated animals was significantly higher than in sham-operated animals at day 7 (P<0.05).

MCP-1 Expression: Real-Time PCR Analysis

For a more accurate quantification of mRNA levels, we performed the sensitive TaqMan real-time PCR. Figure 2 shows a representative study for MCP-1. MCP-1 plasmid was serially diluted, and real-time PCR data were plotted with template copy numbers versus threshold cycle (Ct) values (Figure 2A) or cycle numbers versus ΔRn (ratio of reporter dye emission to quencher dye emission; Figure 2B). The amplification (as indicated by the Ct value) was in a linear relationship with the initial template concentration, and all testing samples were located within range (Figure 2A).

The quantitative data (day 7) for MCP-1, normalized to the housekeeping rpl32 transcript, are shown in Figure 3. The levels of MCP-1 for sham-operated animals (n=5) were

Results

Characterization of the Experimental Model

Table 2 presents selected cardiovascular and renal parameters of control and experimental groups. Rats with decompensated CHF were characterized by a significantly lower UNaV, bradycardia, and a lower systolic blood pressure compared with either compensated or control animals. Also, the heart-to-body weight ratio was significantly higher in rats with decompensated CHF. Similar findings have been reported previously.15 Echocardiography demonstrated that decompensated CHF was associated with a marked degree of cardiac dilatation (Table 2).

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<th>TABLE 2. Selected Cardiorenal Parameters</th>
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<td>Control</td>
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<tr>
<td>BW, g</td>
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<tr>
<td>H/BW, g/100 g BW</td>
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<td>SBP, mm Hg</td>
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<td>ESD, mm</td>
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<td>UNaV, μmol/24 h</td>
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BW indicates body weight; H/BW, heart/body weight ratio; SBP, systolic blood pressure; HR, heart rate; EDD, left ventricular end-diastolic diameter; ESD, left ventricular end-systolic diameter; and Comp and Decomp, rats with compensated and decompensated heart failure, respectively.

*P<0.05 vs control values.
†P<0.05 vs compensated animals.

Figure 1. Representative Northern hybridization to identify MCP-1 mRNA in left ventricle of sham and ACF rats on days 1, 3, and 7. Rpl32 mRNA was used as housekeeping control.
288 ± 22 copies/ng total RNA and increased to 665 ± 90 in animals with ACF (n=10; P<0.001) (Figure 3A). The separate analyses for compensated and decompensated animals (Figure 3B) showed 502 ± 62 copies/ng in compensated animals (n=5) and 826 ± 138 copies/ng in decompensated animals (n=5) (P<0.05 compared with compensated and sham).

Immunohistochemistry
Control experiments did not produce MCP-1 staining. The immunoreactive staining for MCP-1 in sham, day 1, and day 3 ACF animals was barely detectable. In contrast, in day 7 compensated and decompensated animals, MCP-1 immunoreactivity was clearly increased. It was expressed in endothelial cells and vascular smooth muscle of blood vessels, as confirmed by serial staining with factor VIII. Infiltrating cells accounted for only a small fraction of MCP-1-positive interstitial cells, the majority being capillary endothelium and presumably fibroblasts (Figure 4). Macrophage staining was quantified by counting 5 high-power fields (×200). There was a significantly higher macrophage count in decompensated (5.3 ± 1.1) than in compensated (1.9 ± 0.7) or sham animals (1.7 ± 0.7) at day 7 (P<0.05). Strikingly, MCP-1 immunoreactivity was noted in cardiomyocytes of rats with decompensated CHF (Figure 5) but not in cardiomyocytes of control or compensated animals.

Binding Assays
To further assess the significance of the alterations in the MCP-1 system in rats with experimental CHF, binding assays using human recombinant 125I-labeled MCP-1 were carried out. Although human MCP-1 differs from rat MCP-1 in the absence of 25 to 48 carboxy-terminal amino acids, the data clearly demonstrated radioligand binding to the rat heart in a specific manner, in the range of 70% to 80% of the total binding. Competition studies demonstrated a single, high-affinity binding site. Homologous competitive binding data were used to calculate the affinity of recombinant human MCP-1 to the rat myocardium, yielding IC50 values of 0.071 ± 0.015, 0.28 ± 0.06, and 0.14 ± 0.04 nmol/L for sham, compensated, and decompensated animals, respectively (P<0.05) (Figure 6A). The calculated values of the density of...
inflammatory cytokines, in particular TNF-α, is interesting. Such an activation of the MCP-1 system and the deterioration into a decompensated state of CHF, it is interesting to note that such an association has been suggested previously for proinflammatory cytokines, in particular TNF-α. The induction of an inflammatory response in the failing myocardium by agents like MCP-1 or TNF-α may lead to further deterioration in cardiac performance and a consequent transition from a compensated into a decompensated state.

The demonstration of MCP-1 upregulation in the present study is based on several methodological approaches. In addition to Northern hybridization, we used a novel, quantitative real-time PCR technique based on the 5′ nuclease activity of the Taq DNA polymerase. The real-time PCR uses the number of cycles needed to reach a fixed threshold amount of PCR product as a measure of the initial concentration of the target nucleic acid.18,22 The sensitive fluorescence detection system allows the Ct to be observed when PCR amplification is still in the exponential phase, thus avoiding the possibility that reaction components become limiting. The modification of using a cloned plasmid DNA template to obtain a standard curve in addition to a housekeeping gene, as done in these experiments, permits the measurement of the absolute copy numbers of the transcript, allowing the detection and quantification of low abundant target molecules with high accuracy.22

The notion that the MCP-1 system is upregulated in rats with ACF is further supported by the immunohistochemical data. MCP-1 immunoreactivity was localized in endothelial cells, smooth muscle cells, interstitial cells, and infiltrating cells in the myocardium of rats with compensated and decompensated CHF. Strikingly, in decompensated animals, MCP-1 protein was also localized in cardiomyocytes. Previously, it was shown that MCP-1 expression may occur in stimulated neonatal cardiomyocytes in vitro.23,24 Although we cannot exclude the possibility that MCP-1 was adsorbed to the cell surface, the findings of our study suggest that the adult rat cardiomyocyte is capable of expressing MCP-1 in vivo in response to volume overload. Although this is reminiscent of the TNF-α expression in cardiomyocytes of the failing myocardium,3 nothing is known at present about the functional significance of this finding. MCP-1 may serve to guide mononuclear cells to these cardiomyocytes to phagocytize them; conversely, MCP-1 may have more direct effects on the cardiomyocyte, as shown for induction of intercellular adhesion molecule-1 by MCP-1.23 An earlier report on MCP-1 expression in a hypertensive model of heart failure in the rat reported that MCP-1 expression was confined primarily to endothelial cells and infiltrating cells.11 The discrepancy with our results may be due to the underlying cause of cardiomyopathy in the 2 different models.

The overall infiltration of macrophages in decompensated animals was significantly higher than in compensated and sham-operated animals. MCP-1 may play a role in the chemotraction of these monocytes in accordance with the proposed main function of MCP-1 in heart failure, ie, its action on chemotaxis of monocytes, macrophages, and T lymphocytes.11,24–26

The mechanism responsible for the upregulation of MCP-1 in rats with ACF has not been delineated in the present study. Because TNF-α has been shown to induce MCP-1 expression in other systems,5,9 the possibility that TNF-α could contribute to the increase in MCP-1 in our model warrants further study. In addition, this experimental model is characterized by a marked activation of the systemic and intracardiac...
Figure 4. A, C, D, and E are frozen sections of same blood vessel as shown in inset of A in a 7-day decompensated rat. A, Control staining omitting primary antibody showed no evidence of nonspecific staining. B, In a day 7 sham-operated animal, almost no MCP-1 staining is detectable. C, MCP-1 is positive in vascular endothelium (arrowheads) and smooth muscle (arrows) and in small interstitial blood vessels and cells. D, Anti–factor VIII–labeled vascular endothelium alone (arrowheads). E, ED-1 as macrophage marker shows scarce infiltration (arrows). Hematoxylin counterstaining; magnification ×200.
renin-angiotensin system in proportion to the severity of the disease.\textsuperscript{14,15} Indeed, an association between Ang II and induction of MCP-1 gene expression has been documented in rat vascular smooth muscle cells, in aortic tissues of hypertensive rats, and in rat mesangial cells in experimental nephritis.\textsuperscript{12,13,27} In vascular smooth muscle cells, the Ang II–induced MCP-1 mRNA accumulation was mediated by the AT\textsubscript{1} receptor.\textsuperscript{12} Further studies are necessary to test the possibility that such a relationship between Ang II and MCP-1 also exists in the myocardium of rats with ACF.

In addition to overexpression of MCP-1 in the myocardium, our data also suggest that volume-overload CHF in rats is associated with decreased myocardial binding of the chemokine. At present, no data are available on MCP-1 binding properties in cardiac tissues. Human MCP-1 binds predominantly to the chemokine receptor CCR-2.\textsuperscript{28} Although human and rat MCP-1 proteins share only a 55% identity, human MCP-1 exerts biological effects in the rat.\textsuperscript{23} Thus, it was possible to determine the presence of an MCP-1 binding site by use of iodinized human MCP-1 as a ligand. Our data show that with the development of severe heart failure, the MCP-1 binding site was downregulated like other binding sites, such as the \(\beta\)-adrenergic receptor or the TNF-\(\alpha\) receptor.\textsuperscript{3} The mechanism by which the MCP-1 receptor is regulated in our model of heart failure warrants further investigation. The possibility that the receptors were saturated with endogenous MCP-1, which may not be completely washed out during the membrane preparation, cannot be ruled out. However, additional mechanisms have been implicated in the downregulation of the MCP-1 receptor in other systems. In addition to lipopolysaccharides and other microbial agents,\textsuperscript{29} TNF-\(\alpha\), IL-1, and interferon-\(\gamma\) caused a rapid and drastic reduction of CCR-2 mRNA and protein levels in vitro.\textsuperscript{30,31} Thus, cytokines that upregulate MCP-1 may lead to downregulation of MCP-1 receptor and thus modulate the recruitment of monocytes and possibly other actions of MCP-1.
In summary, the findings of the present study indicate that volume-overload CHF in rats with ACF is associated with upregulation of the myocardial MCP-1 system in proportion to the severity of the disease, as well as with downregulation in MCP-1 binding sites. It is suggested that these changes may contribute to myocardial dysfunction and the progression of CHF in rats with ACF.

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