Hypertensive Myocardial Fibrosis and Diastolic Dysfunction
Another Model of Inflammation?

Fumitaka Kuwahara, Hisashi Kai, Keisuke Tokuda, Motohiro Takeya, Akira Takeshita, Kensuke Egashira, Tsutomu Imaizumi

Abstract—Excessive myocardial fibrosis deteriorates diastolic function in hypertensive hearts. Involvement of macrophages is suggested in fibrotic process in various diseased situations. We sought to examine the role of macrophages in myocardial remodeling and cardiac dysfunction in pressure-overloaded hearts. In Wistar rats with suprarenal aortic constriction, pressure overload induced perivascular macrophage accumulation and fibroblast proliferation with a peak at day 3, decreasing to lower levels by day 28. Myocyte chemoattractant protein (MCP)-1 mRNA was upregulated after day 1, peaking at day 3 and returning to insignificant levels by day 28, whereas transforming growth factor (TGF)-β induction was observed after day 3, with a peak at day 7, and remained relatively elevated at day 28. After day 7, concentric left ventricular (LV) hypertrophy developed, associated with reactive fibrosis and myocyte hypertrophy. At day 28, echocardiography showed normal LV fractional shortening but decreased ratio of early to late filling wave of transmitral Doppler velocity, and hemodynamic studies revealed elevated LV end-diastolic pressure, suggesting normal systolic but impaired diastolic function. Chronic treatment with an anti-MCP-1 monoclonal neutralizing antibody inhibited not only macrophage accumulation but also fibroblast proliferation and TGF-β induction. Furthermore, the neutralizing antibody attenuated myocardial fibrosis, but not myocyte hypertrophy, and ameliorated diastolic dysfunction without affecting blood pressure and systolic function. In conclusion, roles of MCP-1-mediated macrophage accumulation are suggested in myocardial fibrosis in pressure-overloaded hearts through TGF-β-mediated process. Inhibition of inflammation may be a new strategy to prevent myocardial fibrosis and resultant diastolic dysfunction in hypertensive hearts. (Hypertension. 2004;43:739-745.)

Key Words: macrophages ■ fibrosis ■ hypertension ■ diastole

Diastolic heart failure accounts for 30% to 50% of heart failure in clinical practice, and hypertensive heart disease is the major cause of this type of heart failure, especially in elderly populations. Excessive myocardial fibrosis is an important pathophysiological process that contributes to diastolic and eventually systolic dysfunction in hypertrophied heart by increasing myocardial stiffness and by reducing pumping capacity. However, at present, no specific therapy is available to improve left ventricle (LV) diastolic dysfunction.

Recently, we have reported that a pressure overload (PO) model created by a suprarenal abdominal aorta constriction (AC) of Wistar rats shows a rapid progression of marked reactive myocardial fibrosis, which expanded from the perivascular to intermuscular interstitium, associated with diastolic dysfunction. In this model, transforming growth factor (TGF)-β plays a causal role in myocardial fibrosis through fibroblast activation, and TGF-β activity blocking ameliorates diastolic dysfunction by preventing myocardial fibrosis. Mechanisms of the TGF-β induction in PO hearts, however, remained unclarified in our previous study.

Macrophage migration is implicated in an initial and significant event for vascular lesion formation by producing inflammatory cytokines and growth factors. In spontaneously hypertensive rats and experimental renovascular hypertensive rats, it was reported that macrophages accumulate in the perivascular space and co-localize with fibroblasts producing collagen during cardiac hypertrophy. Recently, we have shown that PO induces a rapid and transient induction of intercellular adhesion molecule (ICAM)-1 on endothelial cells of the intramyocardial arteries and that macrophages accumulate in the perivascular area adjacent to the arteries expressing ICAM-1. In addition, macrophage recruitment to the vessels is mainly regulated by monocyte chemoattractant protein (MCP)-1. It was demonstrated that MCP-1 induction was associated with adventitial macrophage accumulation in the aortic wall of hypertensive rats treated with a continuous infusion of angiotensin II (Ang II) or norepinephrine. However, a causal relation of MCP-1 induction and macrophage accumulation to myocardial remodeling and cardiac dysfunction remains to be elucidated.
function still remains to be elucidated. A monoclonal neutralizing antibody (NAb) for rat MCP-1 has been shown to effectively prevent macrophage accumulation in the heart of rats with chronic nitric oxide synthase inhibition by the administration of Nω-nitro-L-arginine methyl ester (L-NAME). Therefore, using the NAb, we investigated the roles of MCP-1 and macrophage accumulation in myocardial remodeling and functional changes during cardiac hypertrophy in AC rats.

**Methods**

All procedures were in accordance with institutional guidelines of animal care and treatment. After male Wistar rats (300 to 400 g) had been anesthetized intraperitoneally with pentobarbital (50 mg/kg), AC or sham-operation (sham) was established. Blood pressure was measured in the unrestricted conscious state, as described previously. Unless otherwise indicated, 6 rats were studied in each group for each time point.

**Protocol 1**

**Tissue Preparation and Morphometry**

Rats were euthanized with an overdose injection of intraperitoneal pentobarbital. After perfusion-fixation at 100 mm Hg, the LV was processed for histological and immunohistological studies. To evaluate myocyte hypertrophy and myocardial fibrosis, 3 independent hematoxylin–eosin-stained and 3 Mallory–Azan-stained sections of each rat were scanned and analyzed using a digital image analyzer, respectively. The shortest transverse myocyte diameter was measured in 50 nucleated transverse sections of the myocytes in each tissue section. The percent area of myocardial fibrosis (%myocardial fibrosis) was calculated as previously described. In each rat, >40 small (internal diameter <200 μm) and >10 large (≥200 μm) arteries were examined for perivascular fibrosis and vascular wall thickening (wall-to-lumen ratio).

**Immunohistostaining**

The sections were subjected to immunohistostaining with an antibody for ED-1 (Chemicon International) or MCP-1 (Santa Cruz) and a commercially available detection system (DAKO). Bromodeoxyuridine (BrdU)/vimentin spindle-shaped cells were defined as proliferating fibroblasts by use of double immunostaining with antibodies for BrdU and vimentin (DAKO) and a double immunostain kit (DAKO). The labeled cells were counted at ×200 magnification in 4 independent entire cross-sections of each animal.

**Reverse-Transcriptase Polymerase Chain Reaction Analysis**

Total RNA was extracted from unfixed hearts, and aliquot (5 μg) of total RNA was reverse-transcribed using Ready-to-Go Your Prime-First Strand Beads (Pharmacia Biotech). Equal amount of the resulting cDNA was subjected to polymerase chain reaction (PCR) with a pair of commercially available primers for rat TGF-β1 (Maxim Biotech), rat MCP-1 (BioSource International), or rat β-actin (BioSource International) and a Taq DNA polymerase core kit (Qiagen). PCR conditions were 30 cycles of denaturing at 94°C for 45 seconds, primer annealing at 58°C for 45 seconds, and primer extension at 72°C for 90 seconds. The number of cycles was within the linear range of the PCR amplification of each gene. The PCR products were separated on 2% agarose gel stained with ethidium bromide.

For quantitative analysis, real-time TaqMan reverse-transcriptase polymerase chain reaction (RT-PCR) was performed in triplicate with TaqMan EZ RT-PCR kit (PE Biosynthesis). PCR conditions and the nucleotide sequences of primers and TaqMan probe for TGF-β were as previously described. The sequences of the primers and TaqMan probe for MCP-1 were: forward primer, 5'AGCCGACTTGGGATCAT-3'; TaqMan probe, 5'-TCACCTGCTGCTACTCATTGCAGTAATGC-3'. The MCP-1 and TGF-β expression levels were normalized by the GAPDH level in each sample, and the relative expression changes in each gene were expressed as an n-fold increase relative to sham rats.

**Protocol 2**

For MCP-1 function blocking, rats were treated intravenously with 2 mg/kg per day of NAb (AC+NAb or sham+NAb rats) or subclass-matched control IgG (R&D system; AC+IgG or sham+IgG rats) daily from 1 day before operation to day 28. The NAb (clone C4) was raised against rat recombinant MCP-1, and the ability of this antibody to neutralize MCP-1 activity was confirmed and described previously. In the present study, the same dose of NAb was used as in the previous study.

**Echocardiographic Studies**

At day 28, rats were anesthetized intraperitoneally with ketamine (50 mg/kg) and xylazine (10 mg/kg) and subjected to echocardiographic study. LV mass, LV fractional shortening, and ratio of early to late filling wave (E/A) of transmitral pulse-wave Doppler velocity were measured as described previously.

**Statistical Analysis**

Each quantitative analysis was performed by a single observer in a blind fashion. One-way ANOVA followed by Scheffe F test was performed for the statistical comparisons. A value of P<0.05 was considered significant.

**Results**

**Protocol 1**

**LV and Myocyte Hypertrophy**

A rapid and sustained elevation of mean arterial pressure (MAP) was induced by AC within 1 day, whereas MAP did not change in sham rats (Figure 1). In AC rats, the LV weight/body weight ratio (LVW/BW), a parameter of LV hypertrophy, was significantly increased after day 7, and then LV hypertrophy progressively developed. In AC rats, myocyte hypertrophy assessed by the transverse diameter of cardiac myocytes became evident after day 7 and thereafter developed progressively, whereas myocyte diameter was not changed in sham rats.

**Macrophage Accumulation and Fibrotic Changes**

ED1 macrophages and BrdU proliferating fibroblasts were sparse in sham rats during the observation period. In AC rats, macrophage accumulation was found in perivascular space after day 1 (Figure 2). At day 3, robust increases in macrophages and proliferating fibroblasts were observed in perivascular space; thereafter they returned to lower levels, which were still significantly higher than shams, by day 28. After day 7, proliferating fibroblasts were found not only in perivascular but also in intermuscular space, to a lesser extent.

Azan-stained myocardial fibrosis area was sparse in sham rats. In AC rats, perivascular fibrosis was first observed at day 3. Thereafter, myocardial fibrosis extended from the perivascular to intermuscular interstitium, which was not accompanied by massive myocardial necrosis and replacement fibrosis. At day 28, %myocardial fibrosis reached >20%.
Myocardial MCP-1 and TGF-β Expression

In AC rats, MCP-1 expression was transiently induced after day 1, peaking at day 3, and disappeared by day 28 (Figure 3A and 3B). In contrast, TGF-β induction was observed after day 3, reached the maximum at day 7, and remained moderately increased at day 28. Immunoreactive MCP-1 was observed mainly in the entire vessel wall of the intramyocardial arteries, whereas interstitial cells occasionally showed MCP-1 immunoreactivity (Figure 3C).

Protocol 2

To determine the causal relation of MCP-1 induction to myocardial remodeling, anti-MCP-1 NAb or control IgG was administered every day from 1 day before operation. MAP was similar in AC/IgG and AC/NAb rats at day 28 (188±9 and 179±8 mm Hg, respectively). At day 28, LVW/BW was smaller by 7% in AC+NAb than in AC+IgG rats (2.76±0.17 versus 2.96±0.17, respectively), although the difference was not statistically significant. Control IgG had no effects on these parameters in AC and sham rats (data not shown).

MCP-1 Neutralization and Early Inflammatory Changes

At day 3, the AC-induced macrophage accumulation was almost abolished by NAb (Figures 4A and 5A). Also, NAb remarkably inhibited the AC-induced fibroblast proliferation at day 3. Furthermore, NAb attenuated the AC-induced TGF-β induction at day 7 (Figure 5B). These parameters did not differ between AC and AC+IgG rats (data not shown). Neither macroscopic nor microscopic abnormalities were found in the hearts of sham/IgG, sham/NAb, or AC/IgG rats.

MCP-1 Neutralization and Myocardial Remodeling

At day 28, NAb remarkably prevented AC-induced myocardial fibrosis, resulting in significant reductions in %myocardial fibrosis (Figures 4B and 6A). In contrast, NAb did not affect AC-induced myocyte hypertrophy. Further, vascular wall remodeling was examined in small and large intramyocardial arteries (Figure 6B). NAb markedly prevented perivascular fibrosis and significantly, yet to a lesser extent, attenuated vascular wall thickening in both small and large arteries at day 28. These parameters were similar in AC and AC+IgG rats (data not shown).
Echocardiographic and Hemodynamic Studies

Echocardiographic and hemodynamic studies were performed in sham, AC/H11001 NAb, and AC/H11001 IgG rats at day 28 (Figure 7). Heart rate and MAP were similar among the 3 groups. AC/H11001 IgG rats showed concentric LV hypertrophy associated with a 2-fold increase in LV mass versus sham rats. Although %fractional shortening was similar in AC/H11001 IgG and sham rats, AC/H11001 IgG rats demonstrated decreased E/A of transmural Doppler velocity and elevated LVEDP versus sham rats. NAb ameliorated E/A reduction and LVEDP elevation induced by AC without affecting %fractional shortening. LV mass of AC/H11001 NAb rats was smaller by 8% when compared with AC/H11001 IgG rats, although the changes were not statistically significant.

Figure 4. Immunohistological and histological studies. A, Effects of MCP-1 neutralization on the accumulation of ED1 (brown) and BrdU (brown/vimentin (red) fibroblasts at day 3. Representative immunohistostainings for ED1 (top) and double immunohistostainings for vimentin (red) and in situ labeled BrdU (brown) (bottom) were shown. B, Representative microphotographs of Mallory-Azan stain at day 28.

Figure 5. MCP-1 neutralization and early fibroinflammatory changes. A, Pooled data of the effects of NAb on macrophage count (top) and proliferating fibroblast count (bottom) at day 3. B, Effects of NAb on myocardial TGF-β expression at day 7. Top, Representative photographs of electrophoresis of RT-PCR products for TGF-β and β-actin. Bottom, Quantitative real-time RT-PCR for TGF-β. Relative changes in TGF-β mRNA levels were expressed as an n-fold increase relative to sham rats. Bar=1 SD (n=6).

Figure 6. MCP-1 neutralization and myocardial remodeling at day 28. A, Pooled data of the effects of NAb on myocyte diameter and %myocardial fibrosis. B, Pooled data of the effects of NAb on vascular wall remodeling. Perivascular fibrosis and wall to lumen ratio were examined in large (internal diameter >200 μm) and small (<200 μm) arteries. Bar=1 SD (n=6).
The present study demonstrated that PO induced rapid inductions of myocardial MCP-1 expression and perivascular macrophage infiltration within day 1, peaking at day 3, and preceding myocardial TGF-β upregulation and fibroblast proliferation. NAb for MCP-1 not only inhibited macrophage accumulation but also prevented fibroblast proliferation and TGF-β expression in PO hearts. Also, AC-induced perivascular and interstitial fibrosis, but not myocyte hypertrophy, was remarkably prevented by NAb. Furthermore, NAb attenuated an increase in diastolic stiffness in PO hearts while not affecting systolic function. Because NAb did not change MAP in AC rats, these effects of NAb appeared independent of hemodynamic changes.

Recently, we have shown that the PO heart of AC rats is a cardiac hypertrophy model characterized by a rapid progression of reactive myocardial fibrosis associated with diastolic dysfunction and that TGF-β plays a crucial role in the fibrotic process. Moreover, ICAM-1 was transiently induced on endothelial cells of the intramyocardial arteries after day 1, peaking at day 3, and returning to baseline by day 7. Also, macrophages accumulated in the area adjacent to the arteries showing ICAM-1 expression. Given that the initial fibrotic process was evident in the perivascular space, we hypothesized that myocardial fibrosis is triggered by macrophage-mediated inflammatory process that is generated by the arterial wall in response to PO.

Discussion

The first important finding of this study is that the initial event of myocardial remodeling in PO hearts was manifested by MCP-1 induction and perivascular macrophage accumulation beginning at day 1 (Figures 2 and 3). Both of the changes peaked at day 3, and after MCP-1 expression had declined, the number of macrophages was gradually decreased. Furthermore, immunoreactive MCP-1 was detected mainly in the intramyocardial arterial wall (Figure 3C). NAb was used to investigate a causal relation of induced MCP-1 to the observed changes, because neutralizing antibodies for MCP-1 are very effective tools to block the effects of MCP-1 in animal models of vascular remodeling and glomerulonephritis. In the present study, NAb almost abolished the PO-induced macrophage accumulation (Figures 4 and 5). Thus, it is suggested that perivascular macrophages are recruited by MCP-1 produced in the vessel wall of AC rats. It is interesting that ICAM-1 is induced by AC in the similar time course to that in MCP-1 and that ICAM-1 functional blocking also abolished perivascular macrophage accumulation. Taken together, the concomitant inductions of MCP-1 and ICAM-1 may indicate their coordinated role in myocardial remodeling in PO hearts by enhancing macrophage recruitment.

It was noteworthy that the inductions of MCP-1 expression and perivascular macrophage accumulation occurred preceding those of TGF-β expression and fibroblast proliferation (Figure 2). Both macrophage accumulation and fibroblast proliferation were observed maximally at day 3. Also, macrophages and activated fibroblasts were co-localized in the perivascular space where myocardial fibrosis was first observed. These findings are consistent with those of earlier studies showing co-localization of macrophages and fibroblasts producing collagen in hypertrophied hearts of spontaneously hypertensive rats and renovascular hypertensive rats. Furthermore, NAb experiments revealed that apparent consequences of MCP-1 induction are not only macrophage accumulation but also activation of fibroblast proliferation and TGF-β induction (Figure 5). Because macrophages produce not only TGF-β but also profibrotic cytokines, the production of these factors by accumulated macrophages may be important in the fibrotic processes. Moreover, TGF-β induces a phenotypic change from fibroblasts to myofibroblasts. Activated fibroblasts and myofibroblasts then express TGF-β and other profibrotic substances, which self-amplify ongoing fibrotic tissue formation. The appearance of myofibroblasts was documented during the early phase of myocardial fibrosis in AC rats. Taken together, it is implied that macrophage accumulation plays a crucial role as the upper stream event of myocardial fibrosis in the later phase through TGF-β induction and fibroblast activation. Given that a decreased, yet significant, number of macrophages were still found in the PO hearts in the later phase (Figure 2), it is also possible that the sustained nature of fibrotic process may be related to the activation of the positive feedback loop created by macrophages, activated fibroblasts, and myofibroblasts.

Kolattukudy et al demonstrated that targeted overexpression of MCP-1 gene in the adult mouse heart muscle induces chronic diffuse infiltration of macrophages without macrophage activation and fibrotic change by 100 days old. Recent studies have shown that besides MCP-1 induction, some additional factors are required to activate recruited macrophages and inflammatory process although the precise nature of this mechanism is unknown. Thus, a possible explanation of the lack of the macrophage activation and fibrosis in their model may be the absence of the proinflammatory stimuli, such as pressure overload.
MCP-1 and Vascular Wall Thickening

MCP-1 has been shown to directly activate proliferation of vascular smooth muscle cells (VSMCs). Also, infiltrated macrophages produce various kinds of cytokines and growth factors that activate VSMCs. Thus, it is possible that NAb partially reduced medial thickening in AC rats through the direct effects on VSMCs and the indirect effects secondary to inhibition of macrophage infiltration.

MCP-1 Functional Blocking and Cardiac Function

In hypertensive hearts, excessive myocardial fibrosis and myocyte hypertrophy are implicated in increased myocardial stiffness and impaired LV relaxation, resulting in diastolic dysfunction. In the present study, MCP-1 function blocking ameliorated diastolic dysfunction in PO hearts without affecting MAP and systolic function (Figure 7). NAb did not affect compensatory myocyte hypertrophy either. Also, the decrease in LV mass by NAb was small enough to be explained by the reduction in myocardial fibrosis. Thus, it is suggested that the observed effects of NAb on diastolic dysfunction are caused mainly by the inhibition of the TGF-β-mediated myocardial fibrosis. Also, the incomplete reverse of diastolic dysfunction after NAb treatment may be explained by the remaining LV systolic mechanical overload.

Possible Mechanisms of MCP-1 Induction

The trigger element of MCP-1 induction was not determined in the present study. Our previous study demonstrated that plasma renin activity was not changed in AC rats during the observation period. Thus, in our model, the systemic renin-angiotensin system is not likely for cardiac remodeling. In addition, AC induced perivascular fibrosis, but not myocyte hypertrophy, in the RV free-wall at day 28 (see online-only supplements). The increase in vascular wall tension induced by hypertension is distributed throughout the whole coronary artery tree of both LV and RV, although RV is not exposed to ventricular pressure load. Thus, it is suggested that in this model, coronary arterial hypertension plays a crucial role in reactive myocardial fibrosis, whereas LV systolic mechanical overload is attributable to myocyte hypertrophy, but not myocardial fibrosis.

Recent studies have suggested that arterial hemodynamic stress could activate the proinflammatory communications around the arterial wall, which in turn induce proinflammatory mediators including MCP-1. First, increase in arterial pressure itself is implicated in a strong signal for activation of MCP-1 induction in hypertensive aortic wall, suggesting a direct MCP-1 upregulation by mechanical strain of the arterial wall. Second, production of reactive oxygen species and induction of inflammatory cytokines and growth factors, such as TGF-β, platelet-derived growth factor, and angII, have been shown in hypertensive arterial wall. These factors can upregulate MCP-1 expression. Taken together, MCP-1 induction in PO hearts may occur as a result of an intricate interplay of the mechanical stimulus and the changes in proinflammatory factors derived from the neuroendocrine system, infiltrating inflammatory cells, or vessel wall cells.

1-NNAME-treated rats showed early inflammatory changes such as MCP-1 induction and macrophage infiltration and late coronary arterial hypertrophy and myocardial fibrosis. In 1-NNAME-treated rats, MCP-1 NAb reduced the vascular hypertrophy but did not ameliorate the perivascular fibrosis and cardiac TGF-β induction. Currently, the reason of the discrepancy of the effects of MCP-1 neutralization between AC rats and 1-NNAME–treated rats is unknown. It is likely, however, that the difference of the model is one of the explanations. In 1-NNAME–treated rats, the peak number of infiltrated macrophages was ~16-fold of AC rats. Also, 1-NNAME–treated rats showed not only perivascular fibrosis but also reparative fibrosis associated with myocyte necrosis. These findings suggest that chronic inhibition of nitric oxide synthesis causes chronic and severe myocardial damages. Thus, the underlying mechanisms of myocardial fibrosis may be somewhat different between AC rats and 1-NNAME–treated rats.

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Kuwahara: Macrophages and Cardiac Fibrosis

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Online Only Supplement
Figure I. Mallory-Azan stained sections of the RV-free wall of sham and aortic constricted (AC) rats at day 28. Perivascular fibrosis was observed in the RV-free wall of AC rats, but not in sham rats.

Figure II. Myocyte diameter of the left ventricular (LV) and RV in sham (open) and AC (closed) rats at day 28. Myocyte hypertrophy did not occur in RV in AC rats. Myocyte diameter was measured as described in the “Methods” section. Bar=1SD (n=6).