Role of Monocyte Chemoattractant Protein-1 in Cardiovascular Remodeling Induced by Chronic Blockade of Nitric Oxide Synthesis

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Background—Chronic inhibition of endothelial nitric oxide (NO) synthesis by the administration of N\textsuperscript{ω}-nitro-L-arginine methyl ester (L-NAME) to rats induces early vascular inflammatory changes (monocyte infiltration into coronary vessels and monocyte chemoattractant protein-1 [MCP-1] expression) as well as subsequent arteriosclerosis (medial thickening and perivascular fibrosis) and cardiac fibrosis. However, the role of MCP-1 in this process is not known.

Methods and Results—We investigated the effect of a specific monoclonal anti–MCP-1 neutralizing antibody in rats treated with L-NAME to determine the role of monocytes in the regulation of cardiovascular remodeling. We found increased expression of MCP-1 mRNA in vascular endothelial cells and monocytes in inflammatory lesions. Cotreatment with an anti–MCP-1 antibody, but not with control IgG, prevented the L-NAME–induced early inflammation and reduced late coronary vascular medial thickening. In contrast, the anti–MCP-1 antibody did not decrease the development of perivascular fibrosis, the expression of transforming growth factor (TGF)-β\textsubscript{1} mRNA, or systolic pressure overload induced by L-NAME administration.

Conclusions—These results suggest that MCP-1 is necessary for the development of medial thickening as well as monocyte recruitment. In contrast, the pathogenesis of fibrosis may involve other factors, such as TGF-β\textsubscript{1}. (Circulation. 2000;102:2243-2248.)

Key Words: endothelium-derived factors ■ remodeling ■ growth substances ■ inflammation ■ cell adhesion molecules ■ proteins

A growing body of evidence suggests that endothelium-derived nitric oxide (NO) acts as an antiarteriosclerotic factor.\textsuperscript{1,2} Specifically, inhibition of NO synthesis upregulates cell adhesion molecules and/or monocyte chemoattractant protein-1 (MCP-1) in cultured endothelial cells through increases in oxidative stress and/or the activity of nuclear factor (NF)-κB.\textsuperscript{3–6} We have recently shown that chronic inhibition of NO synthesis with N\textsuperscript{ω}-nitro-L-arginine methyl ester (L-NAME) induces vascular inflammation (monocyte infiltration into the coronary vessels associated with induction of MCP-1) in the early phase and causes arteriosclerosis (medial thickening and fibrosis) of coronary arteries and cardiac fibrosis in the late phase of L-NAME administration in rats.\textsuperscript{7–12} The importance of our observation is supported by the fact that the adhesion of mononuclear cells to and their infiltration into the blood vessel wall are assumed to be crucial early arteriosclerotic events.\textsuperscript{13,14} In addition, it has been shown that the plasma level of endogenous NO inhibitor is increased in patients with arteriosclerosis.\textsuperscript{15,16} These findings suggest that early inflammatory changes may contribute to the development of later arteriosclerotic changes. However, no direct evidence for the role of monocytes in the development of such arteriosclerotic changes exists.

MCP-1, a member of the C-C chemokine family, is a potent chemotactic factor for monocytes.\textsuperscript{17} MCP-1 is produced constitutively, or after induction by oxidative stress, cytokines, or growth factors, by a variety of cell types, including monocytes, smooth muscle cells, and endothelial cells.\textsuperscript{17} Increased expression of MCP-1 mRNA or protein has been observed in animals and humans with arteriosclerosis or atherosclerosis.\textsuperscript{18,19} Accordingly, in the present study, we investigated the role of MCP-1 in vascular inflammation and cardiovascular remodeling in a rat model of chronic inhibition of NO synthesis using a specific monoclonal anti–MCP-1 neutralizing antibody.

Methods

Animal Model of Chronic Inhibition of NO Synthesis

The study protocol was reviewed and approved by the Committee on Ethics on Animal Experiments, Kyushu University Faculty of...
Medicine, and the experiments were conducted according to the Guidelines for Animal Experiments of Kyushu University Faculty of Medicine. Twenty-week-old male Wistar-Kyoto rats were obtained from an established colony at the Animal Research Institute of Kyushu University Faculty of Medicine.

Experiment 1: Four groups of rats were studied. The control group received untreated chow and drinking water. The second group (L-NAME) received L-NAME in the drinking water (1 mg/mL). The third group (L-NAME+MCP-1Ab) received L-NAME in the drinking water and an anti–MCP-1 antibody (2 mg·kg⁻¹·d⁻¹ IV via the tail vein). The anti–MCP-1 monoclonal antibody (clone C4) was raised against recombinant rat MCP-1. Twenty. The specificity of the antibody has been confirmed by immunoprecipitation. The antibody (C4) specifically precipitated the labeled rat MCP-1 secreted by concanavalin A-stimulated rat spleen cells. The neutralizing activity of this antibody has been demonstrated under both in vitro and in vivo conditions. The fourth group (L-NAME+IgG) received L-NAME in the drinking water and an irrelevant isotype-matched IgG (2 mg·kg⁻¹·d⁻¹ IV via the tail vein). On day 3 or 28 of treatment, systolic blood pressure was measured by the tail-cuff method. Venous blood was then collected, and the rats were killed for morphometric, immunohistochemical, or biochemical analysis.

Experiment 2: To determine whether the beneficial effects of the anti–MCP-1 antibody requires the administration of the antibody during the whole 28-day period, 3 groups of rats were studied. The control group received untreated chow and drinking water. The second group received L-NAME in the drinking water for 28 days while an anti–MCP-1 antibody (2 mg·kg⁻¹·d⁻¹ IV via the tail vein) was administered during the first 7 days. The third group (L-NAME+IgG) received L-NAME in the drinking water for 28 days while a control isotype-matched IgG (2 mg·kg⁻¹·d⁻¹ IV via the tail vein) was administered during the first 7 days. The rats were killed for morphometric analysis on day 28 of treatment.

Experiment 3: To determine an appropriate dose of an anti–MCP-1 antibody, we examined the effect of administration of an anti–MCP-1 antibody in several doses on monocyte infiltration into the dermis induced by intradermal injection of recombinant human MCP-1. After rats were injected intravenously with the anti–MCP-1 antibody (0.5 mg·kg⁻¹·d⁻¹ IV), the recipients were injected intradermally into the ear pinna with recombinant human MCP-1 (1 μg/100 μL). The number of positive cells per injected site was counted.

Histopathology and Immunohistochemistry

For histopathological and immunohistochemical analyses, 6 rats in each group were killed on day 3 or 28 of treatment. The heart was perfused via the aorta at a pressure of 90 mm Hg, and the coronary vasculature was fixed with methacarn solution. The heart was excised and cut into 5 pieces perpendicular to the long axis. Tissue was embedded in paraffin and cut into slices 5 mm thick. Sections were mounted on glass slides and stained with hematoxylin–eosin solution or Masson’s trichrome solution.

Sections were immunostained with antibodies against rat monocyte/macrophage (ED1, Serotec), proliferating cell nuclear antigen (PCNA) (Dako), α-smooth muscle actin (Dako), or nonimmune mouse IgG (Zymed). The slides were washed and incubated with biotinylated, affinity-purified goat anti-rabbit IgG (Nitirei) as the secondary antibody. After avidin–biotin amplification, the slides were incubated with 3,3’-diaminobenzidine and counterstained with hematoxylin.

In Situ Hybridization

Four rats in the control and L-NAME groups were killed on day 3 of treatment. Digoxigenin-labeled single-strand RNA probes (sense and antisense) were generated by use of a DIG RNA labeling kit (Boehringer Mannheim) according to the manufacturer’s protocol. Rat MCP-1 probe, a 665- and 930-base fragment of rat MCP-1, was used. In situ hybridization was performed on 2% p-formaldehyde-embedded sections as described.

Figure 1. Time course of systolic blood pressure in control, L-NAME, L-NAME+MCP-1Ab, and L-NAME+IgG groups. *P<0.01 vs control group.

Morphometry and Cell Counting

Morphometry and cell counting were performed by a single observer who was blind to the treatment protocols as described. Each section (5 per heart) immunostained with an antibody against ED1 or PCNA was scanned at ×40 magnification. The number of positive cells in each section was determined, and the values for each section were added together. The average number of positive cells per section was determined for each animal.

To evaluate the thickening of the coronary arterial wall and the extent of perivascular fibrosis, short-axis images of large coronary arteries (internal diameters >200 μm) and small coronary arteries (internal diameters <200 μm) were analyzed. The inner border of the lumen and the outer border of the tunica media were traced from Masson’s trichrome–stained sections at ×100 to ×200 magnification. The wall-to-lumen ratio (the ratio of medial thickness to internal diameter) and the area of fibrosis (area of collagen deposition stained with aniline blue) immediately surrounding the blood vessel were then calculated. Perivascular fibrosis was estimated as the ratio of the area of fibrosis surrounding the vessel wall to the total vessel area. Myocardial reparative fibrosis after myocyte necrosis was also determined. Areas of myocardial necrosis replaced by fibrosis were calculated as the total area of fibrosis in the entire visual field divided by the total area of connective tissue and myocardium in the visual field.

Northern Blot Analysis

Five rats in each group were killed on day 3 of treatment. The hearts were removed, snap-frozen in liquid nitrogen, and stored at −80°C. Total RNA was extracted from each sample by the acid guanidinium thiocyanate–phenol-chloroform method, and poly(A)⁺ RNA was purified with an oligo(dT)-cellulose column. Northern blot hybridization was performed as we described previously. The cDNA probes used included rat TGF-β, rat type I collagen (American Type Culture Collection), and mouse GAPDH (American Type Culture Collection). Relative amounts of TGF-β and collagen mRNA were normalized against the amount of GAPDH mRNA.

Measurements of ACE Activity

Five rats in each group were killed on day 3 of treatment. Cardiac tissue was isolated, and the ACE activity was measured by fluorometric assay as previously described. Tissue ACE activity was calculated as nmol His-Leu generated per mg tissue weight per hour.

Statistical Analysis

Data are expressed as the mean±SEM. Statistical differences were determined by ANOVA and Bonferroni’s multiple comparison test. A level of P<0.05 was considered statistically significant.

Results

Systolic Arterial Pressure

Compared with the control group, the L-NAME, L-NAME+MCP-1Ab, and L-NAME+IgG groups had greater systolic arterial pressures on days 3 and 28 of treatment (Figure 1).
Localization of MCP-1–Producing Cells
We found no evidence of inflammation in the control rats (Figure 2A). In contrast, on day 3, attachment of mononuclear leukocytes to the endothelium of coronary vessels was noted in the L-NAME (data not shown) and L-NAME + IgG (Figure 2A) groups. A marked mononuclear leukocyte infiltration in the perivascular area immediately surrounding the coronary arteries and veins and the myocardial interstitial space was also observed in these 2 groups. The majority of leukocytes that had infiltrated into the lesions were found to be ED1-positive monocytes (Figure 2A). Spindle-shaped α-smooth muscle actin–positive cells (myofibroblasts) were another major cell type (Figure 2A) that was found in the inflammatory lesions. Nuclear staining with PCNA antibody was observed in some endothelial cells, vascular smooth muscle cells in the media, monocytes, and myofibroblast-like cells (Figure 2A). As we previously demonstrated,9,11,12 these inflammatory and proliferative changes were greater in small arteries and veins than in large arteries.

In situ hybridization demonstrated that MCP-1 induction was confined to the endothelium and infiltrating monocytes in the L-NAME and L-NAME + IgG groups (Figure 2B). Staining for the MCP-1 transcript was more intense in small arteries and veins than in large arteries (Figure 2B). In contrast, arterioles and large veins stained weakly (data not shown).

Effects of Anti–MCP-1 Antibody Administration on Monocyte Infiltration Into the Dermis Induced by Recombinant MCP-1
In the rats receiving control IgG, the number of monocytes recruited into the dermis was significantly greater in the areas of MCP-1 injection than in the areas of PBS injection (Figure 3). This increase in ED1-positive monocytes was inhibited in a dose-dependent manner by the intravenous injection of the anti–MCP-1 antibody (0.5, 1.0, and 2.0 mg · kg⁻¹ · d⁻¹). In contrast, treatment with the anti–MCP-1 antibody did not affect infiltration of polymorphonuclear neutrophils into the dermis induced by interleukin-8 (Figure 3). Therefore, the antibody at a dose of 2 mg · kg⁻¹ · d⁻¹ was used for the following experiments.

Effects of Anti–MCP-1 Antibody Administration on Inflammatory and Proliferative Changes on Day 3
When ED1-positive monocytes or PCNA-positive cells were counted, the number of immunopositive cells per section was significantly greater in the L-NAME and L-NAME + IgG groups than in the control group (Figures 2A and 4). The increases in ED1-positive cells and PCNA-positive cells were both markedly reduced by treatment with the antibody against MCP-1 (Figures 2A and 4). In contrast, the antibody did not significantly reduce the appearance of α-smooth muscle actin–positive myofibroblasts (Figure 2A).

Effects of Anti–MCP-1 Antibody Administration on Vascular and Myocardial Remodeling on Day 28
In experiment 1, which examined the effect of the anti–MCP-1 antibody for the 28-day period, the increase in the medial thickening (the wall-to-lumen ratio) of large and small coronary arteries seen in the L-NAME group was significantly inhibited by treatment with the anti–MCP-1 antibody but not by a control IgG (Figures 2C and 5A). In contrast, the increases in perivascular fibrosis and cardiac fibrosis were not affected by the anti–MCP-1 antibody (Figure 5B and 5C).

Expression of TGF-β1 and Type I Collagen mRNA
We examined the expression of TGF-β1 and type I collagen mRNA in the heart (Figure 4). The cardiac TGF-β1 and type I collagen mRNA levels were significantly greater in the L-NAME group. The increased expression of MCP-1 mRNA was not reduced by anti–MCP-1 antibody.

Tissue ACE Activity
Cardiac tissue ACE activity on day 3 was 0.9±0.1 nmol · mg⁻¹ · h⁻¹ in the control group, 1.8±0.2 nmol · mg⁻¹ · h⁻¹ in the L-NAME group (P<0.01 versus the control group), 1.9±0.2 nmol · mg⁻¹ · h⁻¹ in the L-NAME + MCP-1Ab group (P<0.01), and 1.8±0.2 nmol · mg⁻¹ · h⁻¹ in the L-NAME + IgG group (P<0.01).

Discussion
This study demonstrated that treatment with anti–MCP-1 antibody prevents early monocyte infiltration into coronary vessels and myocardial interstitial areas. Furthermore, we showed that anti–MCP-1 antibody treatment inhibits the late development of coronary vascular medial thickening after inhibition of NO synthesis. Our findings suggest that MCP-1 may be essential in the development of coronary vascular medial thickening by recruiting and activating monocytes.

We previously demonstrated in our rat model that the increased angiotensin II activity caused by overexpression of ACE mediates early inflammation with MCP-1 induction and later arteriosclerotic changes in coronary vessels.8,10–12 In the present study, there was no significant difference in the ACE activity between hearts from the L-NAME, L-NAME + MCP-1Ab, and L-NAME + IgG groups. Therefore, it is unlikely that the antibody affected cardiac tissue angiotensin II activity in our experiments. An important feature of MCP-1 induction that emerged in the present study is that the primary cells of MCP-1 transcript induction are the endothelial cells of coronary vessels and the infiltrating monocytes in L-NAME–treated rats. We previously demonstrated that increased MCP-1 gene expression was associated with increased immunoreactivity of MCP-1 not only in the entire vessel wall but also in infiltrating cells and interstitial cells,11,12 indicating that MCP-1 induction in the endothelium and infiltrating monocytes may result in the increase in local production of MCP-1. Although the mechanism of a more intense staining of the MCP-1 transcript in small vessels than in large arteries is unclear, greater induction of MCP-1 transcript in small vessels may explain the greater extent of inflammatory and proliferative changes seen in such small coronary vessels.7–9,12 Thus, the present data strongly suggest
that increased production of MCP-1 plays an essential role in the monocyte infiltration in the inflammatory lesions.

The treatment with anti–MCP-1 antibody also attenuated proliferative changes (the number of PCNA-positive cells) in the present study. PCNA is a nuclear protein that is upregulated from G1 through the M phase of the cell cycle. We show here that PCNA-positive cells include endothelial cells, medial smooth muscle cells, α-smooth muscle actin–positive myofibroblasts, and infiltrating monocytes. Activated monocytes, endothelial cells, and/or smooth muscle cells are capable of producing growth-promoting factors such as platelet-derived growth factor, fibroblast growth factor, and reactive oxygen species. We previously reported that the early inflammatory and proliferative changes as well as increased induction of MCP-1 peaked at day 3 and declined from day 3 to days 7 and 28. Therefore, we hypothesize that locally produced MCP-1 induced the recruitment of monocytes during the early phase and activated vascular smooth muscle cells and monocytes, which in turn caused proliferation of vascular smooth muscle by producing those growth-promoting factors. Treatment with anti–MCP-1 antibody during the 28-day period thereby inhibited the development of vascular medial thickening during the late phase by blocking the biological effects of MCP-1 in monocytes and vascular smooth muscle cells in the present study.

Figure 2. Histopathology, immunohistochemistry, and in situ hybridization. A, Coronary artery sections were stained with hematoxylin-eosin (HE) and were stained immunohistochemically for monocyte/macrophages (ED1), proliferating cells (PCNA), α-smooth muscle actin (α-SMA), and nonimmune IgG (negative control). Sections were harvested on day 3 after L-NAME administration was begun. Bar=20 μm. B, In situ hybridization with a riboprobe for MCP-1 mRNA. Endothelium of coronary arteries and infiltrating cells (possibly monocytes) from a rat receiving L-NAME stain strongly for MCP-1 mRNA with an antisense riboprobe. There is no staining with a sense riboprobe. Thick bar=100 μm. Thin bars=20 μm. C, Coronary artery sections stained with Masson’s trichrome stain from a control rat, a rat receiving L-NAME+IgG, and a rat receiving L-NAME+MCP-1 Ab for 28 days. Bar=100 μm.

Figure 3. Effects of anti–MCP-1 antibody on monocyte infiltration into dermis induced by recombinant MCP-1. Five serial sections were prepared from 1 block of skin tissues injected with PBS, MCP-1, and interleukin-8. Numbers of infiltrating white blood cells in perivascular spaces within 25 μm from vessel wall were counted, and average number of infiltrated cells per section was determined for each animal. *P<0.01 vs PBS; †P<0.05, ††P<0.01 vs control IgG group.
more, early treatment with the antibody during the first 7 days also attenuated the late development of coronary vascular medial thickening, suggesting the importance of MCP-1 induction in the early stage in mediating such vascular structural changes in our model. A recent study demonstrated that MCP-1 may directly stimulate proliferation and migration of cultured vascular smooth muscle cells. Further investigation is needed to clarify the molecular mechanisms responsible for the direct actions of MCP-1 on vascular cells.

Despite the nearly complete inhibition of monocyte infiltration, the anti–MCP-1 antibody could not reduce perivascular and cardiac fibrosis, gene expression of TGF-β1 and type I collagen, or the number of proliferating myofibroblasts. We previously demonstrated that such increases in gene expression of TGF-β1 and fibrosis were prevented by angiotensin II AT1 receptor blockade, suggesting that the upregulation of TGF-β1 and subsequent fibrosis was mediated by increased activity of angiotensin II. Myofibroblasts are usually transformed from interstitial fibroblasts by TGF-β1, and play a central role in the development of tissue fibrosis. Because the antibody almost completely inhibited ED1-positive monocyte infiltration, it is highly unlikely that the dose of the antibody used was insufficient to neutralize MCP-1 activity. These findings suggest that MCP-1 may not block fibrogenesis in our rat model.

As mentioned before, the increased vascular ACE activity plays a key role in mediating early vascular inflammation and later cardiovascular remodeling. Thus, demonstrating the mechanism of vascular ACE activation will provide new insight into how endothelium-derived NO contributes to anti-inflammatory or antiarteriosclerotic properties of the vascular endothelium in vivo. Recently, we were able to show that antioxidant therapy prevents the increase in vascular ACE activity in a rat model of inhibition of NO synthesis, suggesting important roles of oxidative stress not only in inducing vascular NF-κB activation and subsequent MCP-1 expression but also in the pathogenesis of vascular ACE activation. Endothelin-1 and protein kinase C are known to increase ACE activity and are upregulated after blockade of NO synthesis. Therefore, they might contribute to the pathogenesis of vascular ACE activation in our model. Evidence suggests that angiotensin II increases vascular endothelin levels, and vice versa. Further studies are needed to clarify the molecular mechanism of how oxidative stress increases vascular ACE activity.

In conclusion, this study has, for the first time, provided direct in vivo evidence of the essential role of MCP-1 in the development of vascular medial thickening by recruiting and activating monocytes in a rat model of NO synthesis inhibi-
tion. In contrast, the pathogenesis of vascular and cardiac fibrosis involves other factors, such as TGF-β expression and/or pressure overload. Recently, Boring et al. reported that deletion of the CCR2 gene (a receptor for MCP-1) partially suppresses atherosclerotic lesion development in apoprotein E–deficient mice by inhibiting monocyte recruitment, suggesting that the MCP-1/CCR2 pathway is important in the development of atherosclerosis, especially in the setting of hypercholesterolemia. Therefore, anti–MCP-1 treatment appears to be a promising strategy in the prevention and treatment of vascular diseases.

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References
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