Disparity of MCP-1 mRNA and Protein Expressions Between the Carotid Artery and the Aorta in WHHL Rabbits

One Aspect Involved in the Regional Difference in Atherosclerosis

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Objective—This study was designed to examine why in WHHL rabbits, muscular arteries, such as the carotid artery, are relatively resistant to atherosclerosis compared with the aorta, with a special reference to monocyte chemoattractant protein (MCP)-1.

Methods and Results—MCP-1 mRNA expression was quantitated by Northern blot analysis, and its protein expression was quantitated by immunostaining and ELISA at the age of 1, 3, 6, and 12 months (n=5 to 6 each). In the aorta, atherosclerotic lesions were progressively developed with aging, and MCP-1 was highly expressed in endothelial cells and infiltrating macrophages. By contrast, in the carotid artery, atherosclerotic lesions and MCP-1 immunoreactivity were not evident throughout the experimental period. Unexpectedly, however, the extent of MCP-1 mRNA expression was comparable between the aorta and the carotid artery throughout the experimental period. Endothelial cells in primary culture from the aorta and the carotid artery expressed the same extent of MCP-1 mRNA on stimulation by oxidized LDL. There was no abnormality in primary structure of MCP-1 cDNA in WHHL.

Conclusions—These results suggest that in WHHL, the atherosclerosis process, including MCP-1 protein expression, may be reduced in the carotid artery (and possibly in other muscular arteries), accounting in part for the regional resistance to atherosclerosis. (Arterioscler Thromb Vasc Biol. 2003;23:244-250.)

Key Words: atherosclerosis ▪ chemokines ▪ gene expression ▪ monocyte chemoattractant protein-1 ▪ WHHL

Atherosclerosis is a chronic inflammatory disease that is caused by multiple processes, including infiltration of inflammatory cells, proliferation of smooth muscle cells, increase in extracellular matrix, and thrombus formation.1,2 In early atherosclerotic lesions, accumulation of monocytes/macrophages is noted in the intima.1,2 Macrophages accumulate cholesteryl ester and transform into foam cells. Recruited macrophages to the vessel wall promote progression of atherosclerosis by producing and releasing various cytokines, chemokines, and growth factors.1,2

See cover

Watanabe hereditary hyperlipidemic rabbits (WHHL), an animal model of familial hyperlipidemia and atherosclerosis, are known to have severe atherosclerotic lesions in the aorta but not in the muscular arteries.3 However, the mechanism for the regional difference in the susceptibility to atherosclerosis in WHHL remains to be elucidated. It is important to elucidate this mechanism to better understand the mechanisms of atherosclerosis in general and to develop a novel therapeutic strategy for atherosclerosis in particular.

Chemokines attract and activate leukocytes and play an important role in inflammatory processes. Monocyte chemoattractant protein (MCP)-1 is a member of C-C chemokine family and has a potent chemoattractant activity for monocytes/macrophages.4 MCP-1 specifically binds to CC chemokine receptor 2 (CCR2) and fulfills its function.4 Oxidized LDL has been shown to upregulate the expression of MCP-1 in endothelial and smooth muscle cells in vitro.5 MCP-1 is highly expressed in macrophage-rich areas of atherosclerotic lesions in both experimental animals and humans.6 Recent studies have shown that mice deficient in CCR27 or MCP-18...
are resistant to atherosclerosis and that overexpression of mutant MCP-1 suppresses the development of atherosclerosis in apolipoprotein E (ApoE)-KO mice, indicating an important role of MCP-1 in the pathogenesis of atherosclerosis. Thus, it is conceivable that alteration in MCP-1 expression might be involved as one of the mechanisms for the regional difference in susceptibility to atherosclerosis in WHHL.

In the present study, we thus aimed to elucidate the mechanism for the regional difference in susceptibility to atherosclerosis in WHHL with a special reference to MCP-1.

**Methods**

This study was reviewed by the Ethics Committee on Animal Experiment at the Kyushu University and was carried out in accordance with the guidelines for Animal Experiment at the Kyushu University and the Law (No. 105) and the Notification (No. 6) of the Japanese Government.

**Tissue Preparation and Immunostaining**

WHHL rabbits that had been originally developed at Kobe University were obtained and fed a standard chow diet. They were killed at the age of 1, 3, 6, and 12 months with intravenous heparin (1000 U) followed by an overdose of intravenous sodium pentobarbital (25 mg/kg) (n = 5 to 6 each). As a genetic control, Japanese white rabbits (JW) were treated in the same manner. The descending thoracic aorta was divided into 5 sections with an equal length, with a number starting from the most upper portion. Sections 1 and 5 were used for Northern blot analysis, sections 2 and 4 for protein analysis, and section 3 for histological analysis. For mRNA and protein analysis, the aortic sections (sections 1, 2, 4, and 5) were frozen in liquid nitrogen, and for histological analysis, the section 3 was fixed with methacarn solution and embedded in paraffin. The bilateral carotid arteries were prepared in accordance with the guidelines for Animal Experiment at the Kyushu University and was carried out in the use of random priming kit (Takara Shuzo). Total RNA was isolated from frozen arteries or rabbit aortic endothelial cells using ISOGEN (Nippon Gene). Total RNA (20 μg) was electrophoresed on a 1.2% agarose-formaldehyde gels and transferred onto nylon membrane (Hybond N, Amersham). Membranes were hybridized with radiolabeled cDNA probes mentioned above for 8 hours at 42°C. Then the membranes were washed in 2×SSC, 1% SDS at 55°C and were exposed to Kodak XAR5 film for 8 hours with intensifying screens for following autoradiography. Relative amounts of MCP-1 mRNA were normalized against the amount of GAPDH mRNA.

**Cell Culture**

The descending thoracic aorta and carotid artery were excised from WHHL at the age of 5 months and the adventitial adipose tissue was removed, after which the arteries were gently washed with PBS from one end (n = 3). The pieces of the arteries with both ends open were incubated in Dispase solution (1000 U/mL, Sanko Pharmaceuticals) for 30 minutes at 37°C. After being cut open longitudinally, the arterial lumen was gently washed with PBS using a syringe with 23-gauge needle. The harvested cells were then centrifuged at 180g for 10 minutes, and the sedimented endothelial cells were used for primary cell cultures. The sedimented endothelial cells were resuspended in autologous RPMI 1640 (Nissui Pharmaceutical Co) supplemented with L-glutamine (0.294 g/L, Gibco), 20% FBS (Microbiological Associates), epidermal growth factor (5 μg/mL, Wako Pure Chemical), hydrocortisone (1 μg/mL, Becton Dickinson Labware), 15 μg/mL endothelial cell growth supplement (Collaborative Research), and 100 μg/mL heparin. An antibiotic cocktail supplement (containing of 15 μg/mL of gentamycin, 1.5 μg/mL of amphotericin B, 50 μg/mL of ampicillin, and 2 μg/mL of minomycin) was added to the medium for primary culture. At the end of day 2 of culture, the old medium was removed by suction and the culture was replenished with fresh medium containing only gentamycin. The cultures were incubated with 200 μg/mL of minimally oxidized LDL (TBARS >5 to 10 nmol/mg of protein) for 24 hours at 37°C. Then, total RNA was immediately isolated from endothelial cells.

**MCP-1 Protein Measurement by ELISA**

Frozen arteries were homogenized in the extraction buffer (150 mmol/L NaCl, 10 mmol/L Tris-HCl [pH 7.5], 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 10 mmol/L EDTA, and 2 mmol/L PMSF). Then homogenates were sonicated for 5 minutes in ice bath and were centrifuged at 18,000g for 30 minutes at 4°C. Each supernatant was stored at −80°C until ELISA for MCP-1. The details of the ELISA assay and the specificity of the antibody to MCP-1 were confirmed previously.

**Northern Blot Analysis**

Expression of MCP-1 mRNA was evaluated by Northern blot analysis. cDNA probes used were a rabbit MCP-1 cDNA (a generous gift from Drs Mukaida and Matsushima) and mouse GAPDH cDNA (American Type Culture Collection). These cDNA probes were labeled with [α-32P]-dCTP (3000 Ci/mmol, Du Pont-NEN) by the use of random primer kit (Takara Shuzo). Total RNA was isolated from frozen arteries or rabbit aortic endothelial cells using ISOGEN (Nippon Gene). Total RNA (20 μg) was electrophoresed on a 1.2% agarose-formaldehyde gels and transferred onto nylon membrane. Membranes were hybridized with radiolabeled cDNA probes mentioned above for 8 hours at 42°C. Then the membranes were washed in 2×SSC, 1% SDS at 55°C and were exposed to Kodak XAR5 film for 8 hours with intensifying screens for following autoradiography. Relative amounts of MCP-1 mRNA were normalized against the amount of GAPDH mRNA.

**Cloning and Sequencing MCP-1 cDNAs**

A pair of primers (forward, 5'-TCCAGCATGAGGCTCCTCG-3'; reverse, 5'-ACCATTAGAATTCAAGAGG-3') were designed for rabbit MCP-1 based on the previous report for cloning of MCP-1 cDNAs by RT-PCR. The first strand cDNA was synthesized with total RNAs from the aorta and the carotid artery (<1 μg each) by Moloney murine leukemia virus reverse transcriptase with oligo (dT) primers according to manufacturer’s instruction (Pharmacia Biotech), and then PCR was carried out. The cycling conditions were as follows: 3 minutes at 92°C for the initial denaturation followed by 35 cycles of 1 minute at 92°C, 2 minutes at 55°C, 1 minute and 30 seconds at 72°C, and 7 minutes at 72°C for the final extension. These PCR products were cloned into the pCR2.1 vector according to the manufacturer’s instruction (TA Cloning Kit, Invitrogen), and multiple clones were sequenced with the use of M13 forward and reverse primers.

**Statistical Analysis**

All results in the text and graphs are presented as mean±SEM. All results were analyzed by ANOVA followed by Bonferroni’s post-hoc test. P<0.05 was considered statistically significant.

**Results**

**Regional Difference in the Extent of Atherosclerosis in WHHL**

Figure 1 shows morphological changes in the aorta of WHHL and JW at the age of 1, 3, 6, and 12 months (n = 5 to 6 each). In the aorta, no atherosclerotic lesion was noted in JW at all ages, whereas in WHHL, early atherosclerotic lesions were noted as early as 3 months, and the lesion formation was progressively accelerated with aging at 6 and 12 months (Figure 2). In contrast, in the carotid artery, there was no atherosclerotic lesion formation in both strains at all ages (Figure 2).

**Immunostaining for MCP-1**

Immunoreactivity of MCP-1 was noted in endothelial cells and macrophages in early aortic lesions of WHHL (Figure 3) but not in the aorta of JW (data not shown). In the advanced
atherosclerotic lesions of WHHL aorta, MCP-1 immunoreactivity was also noted in smooth muscle cells, in addition to endothelial cells and macrophages (Figure 4). In contrast, in the carotid artery, immunoreactivity of MCP-1 was not evident in either WHHL (Figure 3) or JW (data not shown) at all ages (n=5 to 6 each).

MCP-1 Protein Concentrations
We quantitatively measured the MCP-1 protein concentrations in the aorta and the carotid artery of WHHL by ELISA. At the age of 1 month, there was no significant difference in the MCP-1 protein concentrations (pg/mg protein) between the aorta and the carotid artery (Figure 5A, n=3 each). However, at the age of 6 months, the MCP-1 protein concentrations in the aorta were 10-fold higher than those in the carotid artery (Figure 5A, n=4 each). These results of MCP-1 measurement with ELISA corresponded to those of MCP-1 immunoreactivity (Figures 3 and 4).

MCP-1 mRNA Expression
Northern blot analysis demonstrated that MCP-1 mRNA expression was noted to a comparable extent in the aorta and the carotid artery in WHHL at all ages (Figure 5B), whereas no MCP-1 mRNA expression was detected in the aorta or the carotid artery of JW at any age (n=5 to 6 each, data not shown).

Induction by Oxidized LDL of MCP-1 mRNA in Cultured Endothelial Cells
We measured MCP-1 mRNA expression in cultured endothelial cells from the aorta and the carotid artery. The results demonstrated that on stimulation by oxidized LDL, MCP-1 mRNA expression was induced to a comparable extent in cultured endothelial cells from both the aorta and the carotid artery (Figure 6).

MCP-1 cDNA Sequence
MCP-1 cDNA sequence of the WHHL aorta was examined, including full coding region and part of 5'-UTR sequenced in this study (Figure I, available online at http://atvb.ahajournals.org). MCP-1 cDNA sequence of the WHHL aorta was identical to that of normal rabbits reported in GenBank, indicating that no abnormality is present in the primary structure of MCP-1 cDNA in the aorta of WHHL. Furthermore, there was no difference in the MCP-1 cDNA sequence between the aorta and the carotid artery of WHHL (data not shown).
Discussion

The novel findings of the present study in WHHL were the following: (1) MCP-1 protein expression was significantly greater in the aorta than in the carotid artery, whereas the extent of MCP-1 mRNA expression was unexpectedly comparable between the two arteries; (2) The MCP-1 protein expression, may be suppressed in the carotid artery, accounting in part for the regional resistance to atherosclerosis.

Regional Difference in the Extent of Atherosclerosis in WHHL

In the present study, we first confirmed the previous report that there was a marked regional difference in the extent of atherosclerotic lesions between the aorta and the carotid artery in WHHL. Indeed, accelerated atherosclerotic lesions were developed in the aorta, whereas no or minimal lesions addressed.

![Figure 3. Representative immunostainings for MCP-1 in the aorta (top) and the carotid artery (bottom) of WHHL (n=5 to 6 each). The immunoreactivity of MCP-1 was noted in the aorta, along with the progression of atherosclerosis, whereas in the carotid artery, the MCP-1 immunoreactivity was almost absent throughout the experimental period.](image)

![Figure 4. Representative immunostainings for macrophages (RAM-11), MCP-1, and smooth muscle cells (α-actin) in the aorta of WHHL in early atherosclerotic lesions (3 months, top) and advanced atherosclerotic lesions (6 months, bottom) (n=3 each).](image)
were noted in the carotid artery in WHHL. The aortic atherosclerotic lesions were rich in lipid-laden macrophages and contained smooth muscle cells, especially during the age of 3 to 6 months. The distribution of the lesion-prone and lesion-resistant vascular regions may be related to site-specific hemodynamic effects, differences in arterial cholesterol metabolism, and possibly local differences in structure or function of endothelial cells. Progression of atherosclerosis is accelerated by accumulated macrophages. Thus, chemotaxis that attracts monocytes/macrophages may be an important process for the formation of atherosclerotic lesions.

**Role of MCP-1 in the Pathogenesis of Atherosclerosis**

MCP-1 is synthesized and secreted by several cells present in the vessel wall on various stimuli. MCP-1 has a powerful chemotactic effect on monocytes and has been suggested to play an important modulatory role for monocyte-endothelial interactions under flow conditions in vitro. Expression of MCP-1 mRNA and the presence of MCP-1 protein in endothelial cells, macrophages, and smooth muscle cells have been shown by in situ hybridization and immunohistochemistry in aortic atherosclerotic lesions in rabbits and humans. Fruebis et al observed a significant correlation between macrophage infiltration and extent of MCP-1 expression during early atherosclerosis in WHHL. Their finding supports the view that macrophages are an important source of MCP-1. MCP-1 expressed in activated monocytes/macrophages may establish a positive feedback in atherosclerotic lesions for the recruitment of additional monocytes to atherosclerotic lesions.

Regional Difference in the MCP-1 Protein Expression in WHHL

The present study demonstrated that in WHHL, MCP-1 immunoreactivity was highly detected in endothelial cells, infiltrating macrophages and smooth muscle cells in atherosclerotic lesions of the aorta but not in the carotid artery. The MCP-1 protein concentrations measured by ELISA in the WHHL aorta were significantly higher than those in the carotid artery, a consistent finding with that by immunostaining for MCP-1.

Oxidized LDL may directly injure endothelial cells and thus play an important role in the initiation of adherence and migration of monocytes into subendothelial space. It is known that minimally modified LDL induces MCP-1 in cultured endothelial cells. We examined whether our in vivo

**Figure 5.** A, MCP-1 protein concentrations in the aorta and the carotid artery of WHHL measured by ELISA. The MCP-1 concentrations were significantly increased in the aorta of WHHL at the age of 6 months (n=4) compared with the age of 1 month (n=3), whereas those in the carotid artery remained unchanged. The MCP-1 in the WHHL aorta was mainly present in the intima. P<0.01 vs carotid artery at 6 months or carotid artery and the aorta at 1 month. B, Northern blot analysis for MCP-1 mRNA expression in the aorta and the carotid artery of WHHL (n=3 each). The MCP-1 mRNA expression was normalized by GAPDH mRNA expression. P<0.01 vs 1 month.
finding is also noted in vitro in cultured endothelial cells in response to oxidized LDL. The results indicated that this is the case.

It is possible that in the early stage of atheroma formation, MCP-1 elaborated by the cells in the vascular wall may be important for the monocyte/macrophage accumulation in the intima. Monocyte-derived macrophages and smooth muscle cells are capable to express MCP-1 mRNA. Increased MCP-1 expression in the WHHL aorta with aging may be attributable to macrophage infiltration in the intima. MCP-1 exerts its chemotactic effects on monocytes in circulating blood through specific cell-surface receptors. Thus, it is not possible to know whether the increased MCP-1 protein expression in the aorta of WHHL is the cause of the regional difference in atherosclerosis or simply the result of the process. Nonetheless, our finding on the regional difference in MCP-1 mRNA and protein expressions between the aorta and the carotid artery of WHHL may be important to warrant a caution when examining atherogenic genes using microarray methods.

Possible Mechanism for the Regional Difference in the MCP-1 Protein Expression

We hypothesized two possible mechanisms that may account for the regional difference in the MCP-1 protein expression between the aorta and the carotid artery: first, a different stability of MCP-1 protein in the two arteries attributable to different primary structures, and second, a different enzymatic mechanism in the two arteries affecting the stability of MCP-1 protein. In this study, we cloned and sequenced cDNAs that encode MCP-1 from the WHHL aorta and carotid artery to examine whether there is any difference in the primary structure of MCP-1 that affects the stability of MCP-1 protein. However, the result of MCP-1 cDNA cloning and sequencing demonstrated that the sequences of cDNA from MCP-1 mRNA in the WHHL aorta and the carotid artery were identical, suggesting that the MCP-1 protein expression is reduced in the carotid artery because of reduced protein synthesis, increased degradation, or both. It is conceivable that the protein expressions of other atherogenic molecules such as adhesion molecules are also reduced in the carotid artery of WHHL. Thus, the present results should be interpreted as showing that the altered MCP-1 protein expression in the WHHL carotid artery only reflects one possible aspect of the altered processes for atherosclerosis in this artery.

In additional studies, we tried to elucidate the mechanisms by examining the fate of exogenously applied MCP-1 to the aorta and the carotid artery of WHHL. However, we found that it is technically difficult to demonstrate the degradation process of MCP-1 in vivo. Thus, the mechanisms involved in the regional difference in MCP-1 protein expression remain to be elucidated in a future study.

Limitations of the Study

Several limitations can be mentioned for the present study. First, no data were provided regarding MCP-1 mRNA expression in cultured endothelial cells because of limited availability of the cells. Second, MCP-1 activity was not examined. Indeed, protein quantity should not be always equated with biological activity. Third, MCP-1 mRNA expression was examined as a whole vascular section but not in more detail by vascular layers (eg, intima versus media). Fourth, from a clinical point of view, examination with coronary arteries would strengthen the clinical relevance of this study. Fifth, although many other atherogenic adhesion molecules and cytokines/chemokines other than MCP-1 also play an important role in the pathogenesis of atherosclerosis and may also be involved in the regional susceptibility to atherosclerosis, the mRNA and protein expressions of those molecules were not examined in the present study. All these points remain to be examined in future studies.

In summary, the present study demonstrates that the reduced MCP-1 protein expression in the carotid artery (and possibly in other muscular arteries) may reflect one aspect of the mechanisms for regional difference in atheroma formation between the aorta and the carotid artery in WHHL. The elucidation of the detailed mechanisms involved may be important to establish a new strategy to treat or prevent atherosclerotic vascular diseases.

Acknowledgments

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M57440 (Rabbit MCP-1 mRNA, complete cds)

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