Local Overexpression of Monocyte Chemoattractant Protein-1 at Vessel Wall Induces Infiltration of Macrophages and Formation of Atherosclerotic Lesion Synergism With Hypercholesterolemia

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Abstract—Monocyte/macrophage infiltration to the arterial wall is an initial step in atherosclerosis, and monocyte chemoattractant protein-1 (MCP-1) is thought to play a central role in the recruitment of these cells. In the present study, we examined the role of local expression of MCP-1 at the vessel wall in the initiation and development of atherosclerosis. We transfected the cDNA encoding rat MCP-1 into the vessel wall of the rabbit carotid artery with the use of the hemagglutinating virus of Japan (HVJ)-liposome method. The rabbits were divided into the following groups: (1) those fed normal chow and transfected with MCP-1-HVJ, (2) those fed a high cholesterol diet (1% cholesterol) and transfected with MCP-1-HVJ, and (3) those fed a high cholesterol diet and transfected with control-HVJ. Prescribed diets were started 2 weeks before transfection and were continued for another 2 weeks. In group 1, vascular lesion formation was not found, and anti-rabbit monocyte/macrophage antibody (RAM-11) staining for monocytes/macrophages was negative, although anti-rat MCP-1 antibody (R-17) staining for rat MCP-1 was positive mainly in endothelial cells. Cholesterol feeding increased plasma cholesterol levels to 1801±444 mg/dL in group 2. In group 2, all rabbits displayed neointimal formation with infiltration of RAM-11–positive cells, and a part of the lesion was also positive for Sudan III lipid staining. In group 3, hypercholesterolemia did not induce the infiltration of monocytes/macrophages and subsequent lesion formation in the vessel wall despite definite upregulation of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 on the endothelium. To initiate atherosclerotic changes, local MCP-1 overexpression at the vessel is not sufficient, and activation of other factors induced by hypercholesterolemia is required.

Key Words: monocyte chemoattractant protein-1 ■ hypercholesterolemia ■ atherosclerosis
atherosclerotic event, we performed in vivo gene transfer of the MCP-1 gene to the vessel walls of rabbit carotid arteries.

Methods

Plasmid Vector

Rat MCP-1 cDNA was a generous gift from Dr Teizo Yoshimura (Laboratory of Molecular Immunoregulation, National Cancer Institute—Frederick, Cancer Research and Development Center, Frederick, Md). Rat intercellular adhesion molecule-1 (ICAM-1) cDNA was a generous gift from Dr Tadashi Horiuchi (Molecular Biology Research Laboratory, Tokyo R and D Center, Daichi Pharmaceutical Co). The complete coding site was excised at the EcoRI endonuclease site and cloned into the expression vector pALTER-MAX (Promega) that contains the cytomegavirus early enhancer promoter. Plasmids were grown in Escherichia coli XL1 blue and prepared with a Plasmid Mega Kit (Qiagen). In the present study, pALTER-MAX without any expression insert was used as the control vector.

Preparation of HVJ-Liposomes Containing Plasmids

Hemagglutinating virus of Japan (HVJ)-liposomes were prepared as previously described. Briefly, lipids (phosphatidylserine, phosphatidylcholine, and cholesterol) were mixed in a weight ratio of 1:4:8.2. The lipid mixture was placed in the bottom of a glass tube and dried with a rotary evaporator to form a lipid thin layer. MCP-1 cDNA plasmid, ICAM-1 cDNA plasmid, or control vector plasmid was incorporated into liposomes by shaking and sonication. The lipid mixture was placed in the bottom of a glass tube and dried with a rotary evaporator to form a lipid thin layer. MCP-1 cDNA plasmid, ICAM-1 cDNA plasmid, or control vector plasmid was incorporated into liposomes by shaking and sonication. The liposomes containing plasmids and HVJ were incubated at 4 °C for 10 minutes and then at 37°C for 60 minutes with gentle shaking to place the HVJ protein on the surface of the liposomes. These solutions (MCP-1-HVJ, ICAM-1-HVJ, and control-HVJ) were concentrated by sucrose gradient centrifugation.

Animals

Male Japanese White rabbits (aged 3 months) were purchased from a breeder (SLC, Hamamatsu, Japan) and kept under conventional conditions in our animal facility. Rabbits were divided into following 3 groups: (1) those fed normal chow (Clea) and transfected with MCP-1-HVJ, (2) those fed a high cholesterol diet (containing 1% cholesterol) and transfected with MCP-1-HVJ, and (3) those fed a high cholesterol diet and transfected with control-HVJ. Each group consisted of 6 to 8 rabbits. Furthermore, in an additional 4 rabbits fed normal chow, we transfected the rat ICAM-1 gene together with the MCP-1 gene. Prescribed diets were started 2 weeks before transfaction, and these diets were continued for the following 2 weeks until the rabbits were euthanized. All animal experiments were conducted according to the Guidelines for Animal Experiments at Kobe University School of Medicine.

Surgical Procedures and Gene Transfer to the Vessel Wall

Transfection was performed with the animals anesthetized by intravenous administration of 0.05 mg/g pentobarbital. The left carotid artery was displayed widely by median section of the neck. Blood flow to the common and internal carotid arteries was restored by releasing the clips, and the wound was closed.

Plasma Lipid Analysis

Fourteen days after transfection, venous blood sampling for measurements of plasma lipid levels was performed. Plasma total cholesterol, HDL cholesterol, and triglyceride levels were measured by the enzymatic method.

Lipid Profile of Rabbit Plasma at Euthanasia

<table>
<thead>
<tr>
<th></th>
<th>Group 1 (n=6)</th>
<th>Group 2 (n=8)</th>
<th>Group 3 (n=8)</th>
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</thead>
<tbody>
<tr>
<td>T-chol, mg/dL</td>
<td>45±8</td>
<td>1801±444*</td>
<td>1707±562*</td>
</tr>
<tr>
<td>TGs, mg/dL</td>
<td>61±43</td>
<td>42±24</td>
<td>37±22</td>
</tr>
<tr>
<td>HDL-C, mg/dL</td>
<td>18±3</td>
<td>16±7</td>
<td>22±6</td>
</tr>
</tbody>
</table>

T-chol indicates total cholesterol; TGs, triglycerides; and HDL-C, HDL cholesterol. Values are mean±SE. *P<0.01 vs group 1.

Histological and Immunohistochemical Analysis

Rabbits were euthanized after blood sampling, and the whole length of the left common carotid artery was removed. The middle portion of the common carotid artery (5-mm length) 2 cm proximal to the carotid bifurcation was excised, embedded in OCT compound (Tissue Tek), frozen in liquid nitrogen, and stored in −80°C. Serial 5-μm- to 10-μm-thick cryosections of the carotid artery were provided for histological and immunohistochemical analyses. Ten serial sections with 500-μm intervals were stained with hematoxylin-eosin and Sudan III for histological analysis. The neointimal area was measured by image processing software (NIH Image). For immunohistochemical staining, rabbit antimonyocyte/macrophage antibody (RAM-11) and anti-rat MCP-1 antibody (R-17) were commercially obtained (Dako). Anti-rat ICAM-1 antibody (G-5) was also commercially obtained (Santa Cruz Biotechnology). Anti-rabbit MCP-1 monoclonal antibody was used for detection of rabbit MCP-1. Anti-rabbit intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) monoclonal antibodies (Rb2/3 and Rb1/9, respectively) were kindly provided by Dr M. Cybulsky (University of Toronto, Toronto, Canada). Biotinylated anti-mouse and anti-goat antibodies (Dako) were used for secondary antibodies. Incubation with streptavidin-peroxidase was followed by the addition of the substrate 3,3'-diaminobenzidine.

Statistical Analysis

Data on plasma lipids are expressed as mean±SE. The significance of the difference between group means was analyzed by 1-way ANOVA followed by post hoc tests. Values of P<0.05 were considered statistically significant.

Results

Plasma Lipids

Administration of a high cholesterol diet to rabbits increased plasma total cholesterol levels in groups 2 and 3, whereas there was no difference in lipid profiles between the 2 groups (Table).

MCP-1-HVJ Transfection in Rabbits Fed Normal Chow

In the first group, we transfected the rat MCP-1 gene to the common carotid arteries of rabbits without any dietary modification. Histological studies of the transfected-side and of the nontransfected side of the carotid arteries were performed 2 weeks after MCP-1-HVJ transfection. As in the nontransfected artery, there were no findings of inflammation or intimal thickening at the transfected site of the carotid artery (Figure 1A and 1B). Anti-rat MCP-1 immunostaining yielded strong expression of rat MCP-1 protein in the endothelium but weak expression in the media and the adventitia (Figure 1C). However, RAM-11–positive cells were barely detected at the transfected site (Figure 1D). There was no expression of rabbit MCP-1, ICAM-1, or VCAM-1 at the transfected site (data not shown). Therefore, histological changes associated with inflammation and atherosclerosis did...
not occur in the carotid artery locally transfected with the rat MCP-1 gene.

**MCP-1-HVJ Transfection in Combination With the High Cholesterol Diet**

In the second group, MCP-1 gene transfer to the dietary hypercholesterolemic rabbits was performed by the same protocol that was used in the first group. MCP-1 gene transfection provoked significant neointimal hyperplasia infiltrated by blight cells with small nuclei (Figure 2A). Sudan III staining revealed that the neointimal formation was associated with lipid accumulation (Figure 2B). RAM-11 staining demonstrated abundant infiltrations of macrophages in the lesion (Figure 3A). The RAM-11–positive cells had infiltrated not only the neointima but also the inner area of the media. Rat MCP-1 was detected at the endothelium and the intima (Figure 3B); however, there was no distinct expression of rabbit MCP-1 in the lesion (data not shown). In addition, there were marked expressions of ICAM-1 and VCAM-1 in the endothelium (Figure 3C and 3D).

In the third group, control-HVJ transfection was performed 2 weeks after initiation of the high cholesterol feeding. The high cholesterol diet was continued for another 2 weeks, and then the rabbits were euthanized. Control empty vector did not induce any histological changes in the vascular wall, even in the presence of hypercholesterolemia (Figure 4A). There were no RAM-11– or anti-rabbit MCP-1–positive cells in the carotid artery transfected with control-HVJ (Figure 4B and 4E). We also examined expressions of ICAM-1 and VCAM-1 in the transected area of this group (Figure 4C and 4D). In contrast to the findings for the second group, ICAM-1 and VCAM-1 were both definitely expressed in the endothelium of the carotid arteries of the third group.

In the additional subgroup of rabbits fed normal chow, we transfected the rat ICAM-1 gene together with the MCP-1 gene to assess the importance of expression of adhesion molecules. Although the overexpression of the MCP-1 gene alone did not induce any atherosclerotic changes, simultaneous overexpression of MCP-1 and ICAM-1 in the absence of hypercholesterolemia could induce intimal hyperplasia infiltrated with monocytes/macrophages (Figure 5). However, the neointima induced by simultaneous transfection of MCP-1 and ICAM-1 was modest and was not associated with lipid accumulation.

**Discussion**

In the present study, we showed that gene transfer of rat MCP-1 to the vascular wall of the carotid artery induced early histological changes associated with atherosclerosis in rabbits treated with a high cholesterol diet. Gene transfer of MCP-1 alone in the absence of hypercholesterolemia failed to induce the vascular lesion. The histological changes associated with atherosclerosis were not detected in carotid arteries when rabbits were treated with a high cholesterol diet for 4 weeks in the absence of MCP-1 gene transfer. The vascular lesion developed by MCP-1 gene transfer under hypercholesterolemia is in accordance with fatty streaks or intimal xanthoma, which occurs in the early phase of atherosclerosis.
An increasing amount of evidence has demonstrated the important role of MCP-1 in the initiation and development of atherosclerosis.\textsuperscript{16,17} In addition to the potent chemoattractant action for circulating monocytes, MCP-1 exerts various effects on monocytes/macrophages. MCP-1 induces the expression of a number of proinflammatory genes that may be linked to atherogenesis.\textsuperscript{18} Recent studies using gene-engineered mice have given direct evidence of the essential role of MCP-1 in atherogenesis. The deficiency of the MCP-1 gene has been demonstrated to provide protection from macrophage recruitment and atherosclerotic lesion formation in LDL-deficient mice and apoB transgenic mice.\textsuperscript{19,20} On the other hand, the lack of CC chemokine receptor 2, the receptor for MCP-1, markedly decreased lesion formation in apoE-deficient mice.\textsuperscript{17} In apoE-deficient mice, transplantation of bone marrow cells from mice overexpressing MCP-1 augmented the progression of atherosclerosis by increasing macrophage numbers and lipid accumulation.\textsuperscript{21}

Our finding that MCP-1 gene transfer alone does not evoke the formation of atherosclerotic lesions is in agreement with previous studies using MCP-1 transgenic mice. A transgenic line in which MCP-1 expression is controlled by the mouse mammary tumor virus long terminal repeat, which directs high levels of expression in multiple tissues, showed no evidence of monocyte infiltration in a variety of tissues examined.\textsuperscript{9} In contrast, other models in which MCP-1 expression was controlled by the organ-specific promoters demonstrated monocyte infiltration in the organ.\textsuperscript{22,23} However, MCP-1 expression alone did not cause morphological evidence of inflammation but led to an enhanced inflammatory response on treatment with other stimuli.\textsuperscript{22} Regarding vessels, a recent report by Sata et al\textsuperscript{24} showed that transgenic overexpression of MCP-1 on the endothelium with the use of ICAM-1 promoter failed to provoke macrophage infiltration and vascular lesions. Therefore, it is thought that MCP-1 alone is not sufficient and that interaction with other factors is required for the infiltration of macrophages in the vascular wall, which is the initiation of vascular lesion formation. In the present study, MCP-1 gene transfer provoked the lesion formation of atherosclerosis in the presence of hypercholesterolemia. The recruitment of monocytes to the lesion might depend on the adhesive properties of the endothelial surface. Monocyte adhesion to the endothelial cells is an important early event in atherogenesis and is controlled in part by the expression of adhesion molecules on the surface of endothelial cells.\textsuperscript{1,25,26} The presence of hypercholesterolemia induces an alteration in the expression of a wide variety of adhesion molecules, which results in the enhancement of monocyte/macrophage recruitment.\textsuperscript{27} Among such adhesion molecules, ICAM-1 and VCAM-1 on the endothelial surface are believed to play a central role.\textsuperscript{28} A significant number of atherogenic factors, including adhesion molecules, are stim-

Figure 3. Representative immunohistochemistry of the carotid arteries transfected with MCP-1-HVJ under hypercholesterolemia. A, RAM-11 staining demonstrated abundant infiltrations of macrophages in the lesion. The RAM-11-positive cells were infiltrated into not only the neointima but also the inner area of the media. B, Rat MCP-1 was detected mainly at the endothelium. C and D, In addition, there were marked expressions of ICAM-1 (C) and VCAM-1 (D) at the endothelium. Similar findings were obtained for all (n=8) rabbits of this group. Bar=50 μm.

Figure 4. Representative sections from a rabbit transfected with control-HVJ in the presence of the high cholesterol diet. A, Hematoxylin-eosin staining of the control-HVJ-transfected carotid artery is shown. Control empty vector did not induce any histological changes in the vascular wall, even in the presence of hypercholesterolemia. B, There was no RAM-11-positive cell in the lesion transfected with control-HVJ. C and D, ICAM-1 (C) and VCAM-1 (D) were expressed in the endothelium. E, No anti-rabbit MCP-1-positive cell was detected in the lesion. F, On the other hand, there were prominent expressions of anti-rabbit MCP-1–positive endothelial cells in sections of the carotid artery obtained from a rabbit treated with lipopolysaccharide. Carotid arterial sections were taken from a rabbit 8 hours after an intravenous injection of lipopolysaccharide (50 μg/kg). Bar=50 μm.

Figure 5. A, Simultaneous overexpression of rat MCP-1 and rat ICAM-1 showed intimal hyperplasia infiltrated with RAM-11-positive macrophages. B, Overexpression of rat ICAM-1 was confirmed by G-5 staining. Similar findings were obtained from all (n=4) rabbits of this group. Bar=50 μm.
ulated or upregulated under hyperlipidemic conditions,1,19 and it may be difficult to determine which factor(s) promoted atherogenesis in the MCP-1 gene–transfected vessels in the present study. However, we found that ICAM-1 and VCAM-1 are upregulated in rabbits treated with a high cholesterol diet in either the presence or absence of MCP-1 gene transfer; thus, we determined them to be candidate molecules. It would be helpful to use blocking antibodies to clarify the importance of the simultaneous expression of adhesion molecules. Because such blocking antibodies against rabbit adhesion molecules are currently not available, we instead performed simultaneous transfection of the rat MCP-1 gene and ICAM-1 gene in the absence of hypercholesterolemia. This transfection resulted in neointimal formation in association with macrophage infiltration, although lipid accumulation did not occur. Therefore, it is considered that the upregulation of adhesion molecules is a requisite for the conduction of monocytes/macrophages to the vascular lesion and the subsequent induction of atherosclerotic vascular lesions.

On the other hand, in our experimental protocol, we did not detect any histological changes associated with atherosclerosis in the carotid artery of rabbits receiving a high cholesterol diet without MCP-1 gene transfer. It is well known that hypercholesterolemia alone is sufficient to cause atherosclerosis. However, in the present experimental protocol, hypercholesterolemia alone induced upregulation of ICAM-1 and VCAM-1 on the endothelial cells but did not induce MCP-1 expression. The lack of MCP-1 expression and absence of atherosclerotic lesions in rabbits treated with a high cholesterol diet may be due to the duration of the high cholesterol diet treatment. Therefore, a longer period of high cholesterol feeding would induce in the rabbit carotid artery atherosclerotic changes such as intimal thickening with inflammatory cell infiltration and lipid deposition.29 It is possible that the expression of MCP-1 at the vascular wall of the rabbit carotid artery requires stimulation with hypercholesterolemia for a period longer than that used in the present study. For our study period, MCP-1 gene transfer markedly accelerated the process of atherosclerosis under hypercholesterolemia. Further studies on the time course of MCP-1 expression under hypercholesterolemia are needed.

In conclusion, we demonstrated directly that the local expression of MCP-1 at the vessel wall, when combined with hypercholesterolemia, induces the formation of early atherosclerotic lesions. However, MCP-1 overexpression at the vessel wall is not sufficient for the recruitment of monocytes/macrophages and lesion formation. In addition to MCP-1, activation of other factors, including ICAM-1 and VCAM-1, induced by hypercholesterolemia is needed to yield infiltration of macrophages and the subsequent lesion formation.

MCP-1 can be a target in the treatment of atherosclerosis. A recent study of Ni et al.30 showed that gene therapy with a mutant of MCP-1 effectively blocked native MCP-1 activity and inhibited the formation of atherosclerotic lesions in apoE knockout mice. The present study should provide a clue to aid in the development of an MCP-1–based strategy for the treatment and prevention of atherosclerosis.

References


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