Impact of Monocyte Chemoattractant Protein-1 Deficiency on Cerebral Aneurysm Formation

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Background and Purpose—Recent studies have suggested that chronic inflammation actively participates in cerebral aneurysm (CA) formation. Macrophages accumulate in CA walls and express proinflammatory genes promoting CA progression, but the molecular mechanisms of monocyte/macrophage recruitment into CA walls remain to be elucidated.

Methods—Monocyte chemoattractant protein-1 (MCP-1) expression in experimentally induced CAs was assessed by immunohistochemistry and Western blotting. The role of MCP-1 in CA formation was examined by MCP-1−/− mice and a plasmid DNA encoding a dominant negative mutant of MCP-1 (7ND). MCP-1 expression in human CAs was examined by immunohistochemistry.

Results—MCP-1 expression was upregulated in aneurysmal walls at the early stage of CA formation. MCP-1−/− mice exhibited a significant decrease of CA formation and macrophage accumulation with decreased expression of matrix metalloproteinase-2, -9, and inducible nitric oxide synthase. Immunohistochemistry for the DNA binding form of nuclear factor-kappa B showed nuclear factor-kappa B activation in MCP-1-expressing cells. Blockade of MCP-1 activity by 7ND resulted in the inhibition of CA progression in rats. In human CAs, MCP-1 was also expressed in CA walls.

Conclusions—These data suggest that MCP-1 plays a crucial role in CA formation as a major chemoattractant for monocyte/macrophage. MCP-1 expression in CA walls is induced through nuclear factor-kappa B activation. MCP-1 may be a novel therapeutic target of medical treatment preventing CA progression.

Key Words: animal model ■ CCL-2 ■ cerebral aneurysm ■ macrophage ■ MCP-1

Cerebral aneurysm (CA) is a common disease with a prevalence ranging from 1% to 5% and a major cause of subarachnoid hemorrhage, one of the most severe forms of stroke.1 At present, subarachnoid hemorrhage due to CA can be prevented only by surgical procedures such as clipping or coiling, and there is no effective medical treatment for the inhibition of CA rupture. To develop a novel medical treatment for it, detailed mechanisms of the initiation, progression, and rupture of CA must be elucidated.

The main pathological features of CAs are degenerative changes2 and inflammatory responses3,4 in aneurysmal walls. We previously demonstrated macrophage infiltration in aneurysmal walls in experimentally induced CAs in rats.5 This is in line with the results from pathological studies using human CA samples.4 Macrophages infiltrating into aneurysmal walls secrete matrix metalloproteinase (MMP)-2 and -9, which degrade the extracellular matrix in aneurysmal walls, thus promoting CA progression.5 Therefore, suppression of macrophage recruitment into aneurysmal walls may result in prevention of CA progression and rupture.

Monocyte chemoattractant protein-1 (MCP-1), also known as CC chemokine ligand-2, plays critical roles in monocyte/macrophage recruitment to affected sites in various vascular diseases such as atherosclerosis6–9 and abdominal aortic aneurysm (AAA).10–12 Recently, we demonstrated nuclear factor-kappa B (NF-κB) activation in CA walls in the early stage of CA formation and a substantial contribution of NF-κB to CA.13 Two NF-κB-binding sites are located in the enhancer region of the MCP-1 gene.14 MCP-1 expression is upregulated in a NF-κB-dependent manner in cultured endothelial cells.15 These findings lead us to a hypothesis that MCP-1-mediated macrophage recruitment into arterial walls is a crucial step for CA formation and progression. To test this hypothesis, we examined MCP-1 expression in rodent and human CAs and the role of MCP-1 in CA formation in the present study.

Materials and Methods

Induction of Experimentally Induced Cerebral Aneurysms in Rats
CAs were induced as previously described by Nagata et al.16 After the induction of pentobarbital anesthesia (50 mg/kg intraperitoneally), the left common carotid artery and posterior branches of

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bilateral renal arteries were ligated at the same time with 10-0 nylon in 7-week-old male Sprague-Dawley rats (Oriental BioService, Osaka, Japan). Animals were fed special food containing 8% sodium chloride and 0.12% β-aminopropionitrile (Tokyo Chemical, Tokyo, Japan), an inhibitor of lysyl oxidase that catalyzes the crosslinking of collagen and elastin. Blood pressure was measured by the tail-cuff method. Animal care and experiments complied with Japanese community standards on the care use of laboratory animals.

**Immunohistochemistry and Cell Counting**

Two weeks or 3 months after aneurysm induction, all rats were deeply anesthetized and perfused transcardially with 4% paraformaldehyde. As a control, age-matched male Sprague-Dawley rats were euthanized as described previously. The anterior cerebral artery/olfactory artery (ACA/OA) bifurcation was stripped and subjected to fluorescence immunohistochemistry as previously described. Primary antibodies used are listed subsequently: rabbit polyclonal anti-MCP-1 antibody (Santa-Cruz, Santa Cruz, Calif), mouse monoclonal anti-smooth muscle α-actin antibody (Laboratory Vision, Fremont, Calif), goat polyclonal anti-CD68 antibody (Santa-Cruz), mouse monoclonal anti-NF-κB p65 subunit antibody, which recognizes only DNA-binding form (Chemicon, Temecula, Calif), rabbit polyclonal anti-inducible nitric oxide synthase antibody (Santa-Cruz), and rabbit polyclonal anti-FLAG antibody (Rockland, Gilbertsville, Pa).

To quantify macrophage accumulation, the number of CD68-positive cells was counted in a 100-μm² square area in rats.

**Western Blotting**

Lysates from the rat cerebral artery adjacent to the ACA/OA bifurcation was extracted using Complete Lysis-M (Roche, Indianapolis, Ind). After electrophoresis, transfer to a PVDF membrane and blocking using a ECL plus blocking agent (Amersham, Buckinghamshire, UK), the membrane was incubated with rabbit polyclonal anti-MCP-1 antibody (Santa-Cruz) for 1 hour at room temperature followed by the incubation with horseradish peroxidase-conjugated antirabbit IgG antibody (GE Healthcare, Buckinghamshire, UK). The signal was detected by chemiluminescent reagent (ECL plus Western Blotting Detection System; GE Healthcare). Alpha-tubulin served as an internal control. Densitometric analyses included 5 independent experiments.

**Monocyte Chemoattractant Protein-1 Deficiency Mice and Induction of Experimentally Induced Cerebral Aneurysms in Mice**

MCP-1 deficiency mice (C57/B6;129S4-Ccl2tm1Rol/J) and their littermates were purchased from Jackson Laboratory (Bar Harbor, Maine). At 7 weeks old, CAs were induced as previously described. Briefly, the left common carotid artery was ligated under 2% Fluothane anesthesia. After 1 week, posterior branches of the bilateral renal artery were ligated. Animals were fed the same food used in the rat CA model. Because it takes more time to develop CAs in mice, mice were euthanized at 5 months of aneurysm induction. After Elastica van Gieson (EvG) staining, aneurysm formation at the ACA/OA bifurcation was assessed under a light microscope. IEL disruption refers to a lesion with the discontinuity of the internal elastic lamina (IEL) without apparent outward bulging of the arterial wall representing an early change of CA formation. Aneurysm refers to an obvious outward bulging of the arterial wall with the fragmentation or disappearance of IEL. Three independent researchers assessed the histopathological changes. Some sections were subjected to immunohistochemistry as described previously. Other primary antibodies used are listed subsequently: rabbit polyclonal anti-MMP-2 antibody (Santa-Cruz), goat polyclonal anti-MMP-9 antibody (Santa-Cruz), and rabbit polyclonal anti-inducible nitric oxide synthase (iNOS) antibody (Santa-Cruz).

To quantify macrophage accumulation or NF-κB activation in aneurysmal walls, the number of CD68-positive cells or DNA binding form of NF-κB p65-positive cells was counted in a 50-μm² square area in mice.

Five animals were subjected to real-time polymerase chain reaction. RNA extraction, conversion to cDNA, and real-time polymerase chain reaction were performed as previously described. Beta-actin was used as an internal control. The primer sets used were: forward 5'-ggaggcattcaggttgctgc-3', reverse 5'-acagcttctgggaggacc-3' for MCP-1; forward 5'-tagtggtgtcccttcctc-3', reverse 5'- tgttgctgacatttca-3' for MMP-2; forward 5'-tagtgagttgacteggg-3', reverse 5'-ctctcactgggctaggag-3' for MMP-9; forward 5'-ctgggtcataacacctc-3', reverse 5'-cattggaaggaagttg-3' for iNOS; and forward 5'-cactaggacaggatacaggga-3', reverse 5'-ccttaagggcaaccttg-3' for β-actin. The second derivative maximum method was used for crossing point determination using LightCycler Software 3.3 (Roche, Basel, Switzerland).

**7ND Treatment**

FLAG-tagged human mutant MCP-1 (7ND) plasmid was constructed and its dominant negative activity was verified in vivo as previously described. Immediately after CA induction and 2 weeks after CA induction, 500 μg of 7ND plasmid or empty plasmid (mock) was injected into the rat femoral muscle. One month after CA induction, rats were euthanized as described previously and subjected to EvG staining and immunohistochemistry for FLAG, MMP-2, MMP-9, and iNOS. Aneurysm size was calculated as the mean of the maximal longitudinal diameter and the maximal transverse diameter.

**Immunohistochemistry for Human Samples**

Human CA samples were obtained from 7 patients who underwent neck clipping for unruptured CAs with informed consent. As a control, we used the middle cerebral artery (n=4) obtained at the superficial temporal artery–middle cerebral artery bypass surgery. Immunohistochemistry for human samples was performed as previously described. For double staining, slides were incubated with primary antibodies for smooth muscle α-actin (DAKO, Carpinentina, Calif), von Willebrand factor (DAKO), and CD68 (DAKO) for 30 minutes at room temperature followed by the incubation with alkaline phosphatase-labeled secondary antibodies and Fast red solution (Sigma Aldrich, St. Louis, Mo).

**Statistical Analysis**

Data (mean±SD) were analyzed by use of Mann–Whitney U test for a 2-group comparison and Kruskal-Wallis one-way analysis of variance on ranks followed by Turkey-Kramer test for multiple comparison. The incidence of aneurysmal changes was analyzed by use of Fisher exact test. Differences were considered statistically significant at P<0.05.

**Results**

**Expression of Monocyte Chemoattractant Protein-1 and Macrophage Infiltration in Rat Aneurysmal Walls**

Two weeks after aneurysm induction, MCP-1 was mainly expressed in the intima, although a few positive signals were observed in the media (Figure 1D). Three months after aneurysm induction, MCP-1 expression was also observed in the adventitia (Figure 1F). MCP-1 expression was localized in CA walls (Figure 1H). In the control arterial wall, MCP-1 expression was only slightly detected (Figure 1B). Western blotting showed the increase of MCP-1 protein expression with CA progression (0.5 month versus 3 months, P<0.01, n=5 per group; Figure 1J). The number of macrophages accumulating in aneurysmal walls increased with CA progression (0 month 0.50±0.84/100 μm² square, n=6; 0.5 month 3.17±2.14/100 μm² square, n=6; 3 months 5.86±2.17/100 μm² square, n=14). The number of macrophages after 2 weeks of aneurysm induction was significantly increased compared with before aneurysm induction (0 month versus 0.5 month:...
Figure 1. MCP-1 expression in experimentally induced CAs in rats. A, C, E, G, EvG staining of the ACA/OA bifurcation of a rat before (0 M; A), 2 weeks (0.5 M; C), and 3 months after aneurysm induction (3 M; E, G). B, D, F, H, Immunohisto staining of MCP-1 (green) of the serial section as A (B), C (D), E (F), and G (H). Red color showed immunohisto staining of smooth muscle α actin. Luminal side is indicated by an arrow. Bar=50 μm. I–J, Western blotting for MCP-1. A representative image of 5 independent experiments is shown. K, The number of CD68-positive cells in a 100-μm square area around CAs. Data were analyzed by the Kruskal-Wallis one-way analysis of variance on ranks followed by Turkey-Kramer test.
P=0.023; Figure 1K). In double immunohistochemistry, the staining of MCP-1 and endothelial nitric oxide synthase were merged, showing the MCP-1 expression in endothelial cells (Figure 2C). A few smooth muscle α-actin-positive cells were also stained by the anti-MCP-1 antibody (Figure 2D). Double immunohistochemistry for MCP-1 and the DNA binding form of the NF-κB p65 subunit showed that NF-κB activation occurred in MCP-1-expressing cells (Figure 2E).

Cerebral Aneurysm Formation in MCP-1−/− Mice
After 5 months of aneurysm induction, only one of 10 MCP-1−/− mice (10%) presented an IEL disruption, whereas 9 of 15 MCP-1+/+ mice (60%) developed aneurysmal changes, including IEL disruption, 3 of which had aneurysms (Figure 3A). The incidence of aneurysmal change, including IEL disruption, was significantly lower in MCP-1−/− mice than in MCP-1+/+ mice (P=0.018). Systemic blood pressure significantly raised after aneurysm induction in both MCP-1+/+ mice (0 month 91.5±17.7 mm Hg, n=10; 5 months 134.7±14.5 mm Hg, n=10; P<0.01) and MCP-1−/− mice (0 month 89.9±19.2 mm Hg, n=10; 5 months 130.8±15.6 mm Hg, n=15; P<0.01), but there was no significant difference between MCP-1+/+ and MCP-1−/− mice (Figure 3B). There was no anatomic difference of the circle of the Willis between MCP-1+/+ and MCP-1−/− mice (data not shown).

The number of CD68-positive cells in MCP-1−/− mice (0.60±0.84/50 cells μm square, n=10) was much smaller than that in MCP-1+/+ mice (5.4±1.6/50 cells μm square, n=14; P<0.01; Figure 3C–H).

Nuclear Factor-Kappa B Activation in MCP-1+/+ and MCP-1−/− Mice
In both MCP-1+/+ and MCP-1−/− mice, the NF-κB p65 subunit was highly activated in arterial walls at the ACA/OA bifurcation (Figures 3I and J) and there was no statistically significant difference in the number of cells with NF-κB activation between the 2 groups (MCP-1+/+ 5.3±2.3 cells/50 μm square, n=6; MCP-1−/− 6.0±1.9 cells/50 μm square, n=6; P=0.63; Figure 3K).

Figure 2. Double immunohistochemistry. A, EvG staining of ACA/OA bifurcation of a rat 2 weeks after aneurysm induction. Luminal side is indicated by an arrow. B, Immunohistostaining of MCP-1. (C–E) Double immunohistostaining of MCP-1 (green) and endothelial nitric oxide synthase (C), smooth muscle α-actin (D), or DNA binding form of NF-κB p65 subunit (E) in a rat 2 weeks after aneurysm induction. Bar=50 μm.
Figure 3. CA formation, macrophage recruitment, and NF-κB activation in MCP-1<sup>−/−</sup> mice 5 months after aneurysm induction. A, CA formation in MCP-1<sup>+/+</sup> and MCP-1<sup>−/−</sup> mice. Data were analyzed by use of Fisher exact test. B, Blood pressure before (0 M) and 5 months after (5 M) aneurysm induction in MCP-1<sup>+/+</sup> and MCP-1<sup>−/−</sup> mice. Data were analyzed by the Mann–Whitney U test. C, A schematic drawing indicating the location of the image (D–G) shown by the square. Immunohistochemistry for CD68 at ACA/OA bifurcation in MCP-1<sup>+/+</sup> (D) and MCP-1<sup>−/−</sup> mice (E). EvG staining of the serial section of D (F) and E (G). Luminal side is indicated by an arrow. Bar=20 μm. H, The number of CD68-positive cells in MCP-1<sup>+/+</sup> and MCP-1<sup>−/−</sup> mice. Data were analyzed by the Mann–Whitney U test. ** P<0.01. I–J, Immunohistostaining of DNA binding form of NF-κB p65 subunit in aneurysmal walls in MCP-1<sup>+/+</sup> (I) and MCP-1<sup>−/−</sup> mice (J). Bar=20 μm. K, Quantification of the cell number of NF-κB activation in MCP-1<sup>+/+</sup> and MCP-1<sup>−/−</sup> mice. Data were analyzed by the Mann–Whitney U test.
Expression of Monocyte Chemoattractant Protein-1, Matrix Metalloproteinase-2, Matrix Metalloproteinase-9, and Inducible Nitric Oxide Synthase in MCP-1+/+ and MCP-1−/− Mice

Immunohistochemistry confirmed that MCP-1 was not expressed in MCP-1−/− mice (Figure 4B). The expression pattern of MCP-1 in MCP-1+/+ mice 5 months after aneurysm induction was similar to that in rats 3 months after aneurysm induction (Figure 4A). Expression of MMP-2, MMP-9, and iNOS mRNA was highly upregulated in aneurysmal walls of MCP-1+/+ mice after aneurysm induction and the expression level of all these mRNAs were significantly lower in MCP-1−/− mice than in MCP-1+/+ mice (MMP-2, P=0.020, n=5; MMP-9, P<0.01, n=5; iNOS, P<0.01, n=5; Figure 4L–N). In immunohistochemistry, all these molecules were abun-
dantly expressed in aneurysmal walls of MCP-1-/- mice 5 months after aneurysm induction (Figures 4C, E, and G), whereas only a small amount of these molecules were expressed in MCP-1-/- mice (Figures 4D, F, and H).

Effects of Monocyte Chemoattractant Protein-1 Blockade by 7ND on Cerebral Aneurysm Progression in Rats

In 7ND-treated rats, immunohistochemistry for FLAG showed a large number of positive signals in cerebral arteries, demonstrating incorporation of the dominant negative mutant of MCP-1 in cerebral arteries (Figure 5A–B). Aneurysm size (C), number of macrophage accumulation (D), and systemic blood pressure (E) 1 month after CA induction in 7ND-treated rats and control rats. Data were analyzed by Mann–Whitney U test. F–M, Immunohistochemistry for MMP-2 (G, K), MMP-9 (H, L), and iNOS (I, M) in the control rat (G–I) the 7ND-treated rat (K–M) 1 month after aneurysm induction. EvG staining of the serial section (F, J). Bar=20 μm. Red color showed immunohistostaining for smooth muscle α-actin. Luminal side is indicated by an arrow.

Figure 5. Effects of 7ND treatment on CA progression in rats. A–B, Immunohistochemistry for an anti-FLAG antibody in cerebral arterial walls 1 month after aneurysm induction (B). EvG staining of the serial section (A). Luminal side is indicated by an arrow. Bar=20 μm. Aneurysm size (C), number of macrophage accumulation (D), and systemic blood pressure (E) 1 month after CA induction in 7ND-treated rats and control rats. Data were analyzed by Mann–Whitney U test. F–M, Immunohistochemistry for MMP-2 (G, K), MMP-9 (H, L), and iNOS (I, M) in the control rat (G–I) the 7ND-treated rat (K–M) 1 month after aneurysm induction. EvG staining of the serial section (F, J). Bar=20 μm. Red color showed immunohistostaining for smooth muscle α-actin. Luminal side is indicated by an arrow.
after aneurysm induction, but there was no significant difference between the control group (148.3±23.3 mm Hg, n=8) and the 7ND-treated group (142.7±24.0 mm Hg, n=8; Figure 5E). In immunohistochemistry, upregulated expressions of MMP-2, MMP-9, and iNOS were inhibited by 7ND treatment (Figure 5F–M).

Monocyte Chemoattractant Protein-1 Expression in Human Cerebral Aneurysms

In human CAs, IEL completely disappeared (Figure 6C) and MCP-1 was expressed, especially in the endothelial cell layer, although some MCP-1-positive cells were also detected in the media (Figure 6A). In a normal middle cerebral artery, MCP-1 expression could not be detected by immunohistochemistry (Figure 6B). Double immunohistochemistry revealed MCP-1 expression in endothelial cells (Figure 6E), smooth muscle cells (Figure 6F), and macrophages (Figure 6G).

Discussion

A growing body of evidence indicates that chronic inflammation contributes to the pathophysiology of CA formation. Various kinds of inflammatory cells, especially macrophages, accumulate in human cerebral aneurysmal walls and in aneurysmal walls of experimentally induced CAs in rats.5 Tolylsam, a selective inhibitor for MMP-2 and MMP-9, inhibited CA progression without alteration of macrophage accumulation in aneurysmal walls.5 Macrophages also express iNOS in aneurysmal walls, and iNOS induces apoptosis in medial smooth muscle cells.29 Extracellular matrix degradation and apoptosis in medial smooth muscle cells result in thinning of aneurysmal walls, promoting CA enlargement and rupture. In MCP-1-/- mice, expression of MMP-2, MMP-9, and iNOS was not upregulated after aneurysm induction. Inhibition of macrophage recruitment into aneurysmal walls leads to a blockade of all these exacerbating factors, which may provide a clue to an effective treatment preventing CA progression and rupture. Another plausible role of MCP-1 in CA progression is the effect on vascular smooth muscle cells. Recent investigations demonstrate that MCP-1 regulates proliferation of vascular smooth muscle cells in neointimal hyperplasia after balloon injury and vein graft intimal hyperplasia.31 It is possible that MCP-1 secreted from endothelial cells might affect vascular smooth muscle cells as a paracrine factor and activate vascular smooth muscle cell to produce cytokines or MMPs.
Recently, we demonstrated that NF-κB was activated in the early stage of CA formation and that NF-κB decoy oligodeoxynucleotide suppressed CA formation. NF-κB decoy oligodeoxynucleotide inhibited the upregulated expression of MCP-1 in aneurysmal walls. In the present study, NF-κB activation occurred in MCP-1-expressing cells (Figure 2D). In MCP-1−/− mice, NF-κB was activated in arterial walls of CA-prone sites (Figure 3F–G), indicating that NF-κB regulates MCP-1 expression in CA walls. MCP-1 expression is induced by a variety of stimuli, including cytokines and shear stress, and induced expression of MCP-1 strongly depends on activation of NF-κB, whereas basal transcription is regulated by SP-1. CA tends to be formed at the arterial bifurcation, which is subjected to high hemodynamic stress. Although the promoter region of MCP-1 contains the cis element directly responding to shear stress, our results indicate that the induced expression of MCP-1 in CA walls is NF-κB-dependent.

Finally, we examined the therapeutic effect of MCP-1 blockade by a dominant negative DNA of MCP-1 (7ND). Transfection of 7ND into skeletal muscle is an established strategy for anti-MCP-1 gene therapy. Cells infected with 7ND secrete 7ND protein into circulating blood, and the 7ND protein binds to the MCP-1 receptor on monocytes or target cells in remote organs, thus blocking the signal of MCP-1. Such blockade of MCP-1 activity suppresses atherosclerosis, vascular inflammation induced by chronic inhibition of endothelial nitric oxide synthase, and neointimal formation after balloon injury. 7ND treatment suppressed CA enlargement with decreased macrophage accumulation and reduced expression of MMP-2, MMP-9, and iNOS in the rat model (Figure 5), suggesting that 7ND may be a promising treatment strategy for prevention of CA progression and rupture.

Taken together, MCP-1 expression was upregulated in the cerebral arterial walls of early aneurysmal changes, especially in the endothelium in an NF-κB-dependent manner. MCP-1 deficiency resulted in the attenuated CA formation with decreased macrophage recruitment and inhibited induction of MMP-2, -9, and iNOS genes. These data suggest that MCP-1 plays important roles in CA formation by recruiting monocytes/macrophages into aneurysmal walls. MCP-1 may be a promising therapeutic target for a medical treatment preventing CA progression and subsequent subarachnoid hemorrhage.

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Disclosures
None.

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