Granulocyte-Macrophage Colony-Stimulating Factor Enhances Leptomeningeal Collateral Growth Induced by Common Carotid Artery Occlusion

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Background and Purpose—Granulocyte-macrophage colony-stimulating factor (GM-CSF) has been reported to accelerate collateral growth (arteriogenesis) at the circle of Willis in rat brain. However, the effect of GM-CSF on leptomeningeal collateral growth has not been established. We examined the effect of unilateral common carotid artery (CCA) occlusion and GM-CSF treatment on leptomeningeal collateral growth in mice.

Methods—Adult mice were subjected to unilateral CCA occlusion or sham surgery followed by an alternate-day regimen of GM-CSF (20 μg/kg) or saline injection. On day 7, latex perfusion was performed in 1 set of mice to visualize the leptomeningeal vessels, and the number of Mac-2\(^+\) monocytes/macrophages on the dorsal surface of the brain was counted. In another set of mice, on day 7, permanent ipsilateral middle cerebral artery (MCA) occlusion was performed, and infarct volume was measured.

Results—Leptomeningeal collateral growth was observed after CCA occlusion, and that was enhanced by GM-CSF treatment. An increase in the number of Mac-2\(^+\) cells on the surface of the brain occurred after CCA occlusion and was enhanced by GM-CSF treatment. Seven days after CCA occlusion, GM-CSF treatment decreased the infarct size attributable to subsequent MCA occlusion.

Conclusion—After CCA occlusion, GM-CSF treatment enhanced leptomeningeal collateral growth and decreased the infarct size after MCA occlusion in mice. (Stroke. 2008;39:1875-1882.)

Key Words: chronic ischemia ■ collateral perfusion ■ focal ischemia

Leptomeningeal collateral growth plays a key role in compensation of chronic cerebral ischemia. In chronic hypoperfusion, collateral flow via leptomeningeal vessels that require time to develop is recruited if the circle of Willis cannot maintain adequate cerebral blood flow (CBF).\(^1\) In acute middle cerebral artery (MCA) occlusion, the presence of leptomeningeal collateral circulation decreases the infarct size and improves clinical outcome.\(^2\) However, there is no completely adequate experimental model for the investigation of leptomeningeal collateral growth. Therefore, the mechanisms underlying leptomeningeal collateral growth remain to be elucidated.

Bush et al\(^3\) reported that 3-vessel (1 carotid plus both vertebral arteries) occlusion induces collateral growth at the circle of Willis in rat brain. Using the 3-vessel occlusion model, monocyte/macrophage lineage was shown to be associated with collateral growth, and granulocyte-macrophage colony-stimulating factor (GM-CSF) was shown to enhance collateral growth.\(^4\) However, leptomeningeal collaterals were not changed in this rat model.

We recently reported that a chronic decrease in cerebral perfusion induced by unilateral common carotid artery (CCA) occlusion in mice results in preservation of cortical perfusion and attenuation in infarct size after ipsilateral middle cerebral artery (MCA) occlusion.\(^5\) However, early after CCA occlusion, the infarct size after subsequent MCA occlusion was not reduced. These results suggested that chronic unilateral CCA occlusion in mice may induce collateral growth at distal leptomeningeal anastomosis. In the present study, we examined the effect of unilateral CCA occlusion on leptomeningeal collateral development and examined the effect of GM-CSF treatment after CCA occlusion on leptomeningeal collateral growth and on infarct size after subsequent MCA occlusion.

Materials and Methods

Animals

Adult male C57BL/6 strain mice (ages 12 to 16 weeks; Charles River Laboratories Japan Inc, Yokohama, Japan) were used in this study.
Mice were given free access to food and water before and after all procedures. In the first experiment, the effects of CCA occlusion on infarct size after subsequent MCA occlusion and on leptomeningeal collateral growth were examined. Animals were assigned to 1 of 3 procedures: sham CCA occlusion (Sham group), 7 days of CCA occlusion (7 days group), and 14 days of CCA occlusion (14 days group). In the second experiment, the effects of GM-CSF treatment 7 days after CCA occlusion on leptomeningeal collateral growth and on infarct size after subsequent MCA occlusion were examined. Animals were assigned to 1 of 4 procedures: sham CCA occlusion followed by injection of saline (sham-saline group), sham CCA occlusion followed by injection of GM-CSF (sham-GM-CSF group), left CCA occlusion followed by injection of saline (CCA-saline group), and left CCA occlusion followed by injection of GM-CSF (CCA-GM-CSF group). The experimental protocol was approved by the Committee for Institutional Animal Care and Use of the Osaka University Graduate School of Medicine.

**Common Carotid Artery Occlusion**

General anesthesia was induced with 4.0% halothane and maintained with 1.0% halothane with an open facemask, and rectal temperature was monitored and maintained at 37±0.5°C with a heat lamp and mat. The left CCA was exposed and ligated with a silk suture. Sham CCA occlusion was performed with a silk suture without ligation.

**Measurement of Relative Cerebral Perfusion by Laser-Doppler Flowmetry**

We have reported that CCA occlusion induces a decrease in cerebral perfusion over the ipsilateral MCA area. However, the effect of CCA occlusion over the ipsilateral anterior cerebral artery (ACA) area is not elucidated. Under general anesthesia, a polyacryl column with an inner diameter of 0.8 mm was attached to the skull 0.5 mm or 3.5 mm lateral to bregma with dental cement, for assessment of the ACA or MCA area, respectively. Percent decrease of cerebral perfusion over the unilateral ACA or MCA area was measured 15 minutes after CCA occlusion.

**Injection of Granulocyte-Macrophage Colony-Stimulating Factor**

Mice were injected subcutaneously with 20 μg/kg GM-CSF (R&D Systems Inc, Minneapolis, Minn) diluted in 0.1 mL saline every second day subsequent to CCA occlusion until 7 days after CCA occlusion. Vehicle-treated mice were injected 0.1 mL saline.

**Middle Cerebral Artery Occlusion Subsequent to Common Carotid Artery Occlusion**

The left MCA was occluded by electrocoagulation as described previously 7 or 14 days after CCA occlusion. Under general anesthesia, mice were placed in the recumbent position, and a vertical skin incision was made at the midpoint between the left orbit and the external auditory canal. The mandible was pulled downward immersed in Zamboni solution (2% paraformaldehyde and 0.2% picric acid) for 2 days. Photographs of the top of the brain were taken at ×50 magnification to measure the vessel diameter of the leptomeningeal anastomosis, and photographs of the bottom of the brain were taken at ×20 magnification to measure the vessel diameter of the circle of Willis. The distal MCA was identified from its branch angle and distinguished from the distal ACA or PCA. The diameter of the leptomeningeal anastomosis was measured at the point of confluence between the distal MCA and the distal ACA or between the distal MCA and the distal posterior cerebral artery (PCA). The diameter of the circle of the Willis was measured at the left ACA just proximal to the point where the olfactory artery diverges. The measurements were done by an individual (T.S.) blinded to the operation and treatment.

**Measurement of Infarct Volume**

Infarct volume was evaluated in 8 1-mm-thick coronal sections stained with 0.8% 2,3,5-triphenyltetrazolium-chloride/saline (TTC) as described previously. The infarct area was measured with NIH Image v. 1.61 software. Infarct volume was determined by integrating the infarct area of the eight sections.

**Immunohistochemistry**

To assess the relationship between collateral arterial growth and monocyte/macrophage accumulation, specimens of latex-perfused...
brain were used for immunostaining with anti–Mac-2 monoclonal antibody and positive cells were quantified on the dorsal surface of the brain. Sagittal 50-μm-thick floating sections between 1.5 mm and 3.0 mm lateral from the midline were prepared with a vibratome. Sections were incubated with anti–Mac-2 monoclonal antibody (1:200; Cedarlane Laboratories Ltd) diluted in Tris-buffered saline at 4°C overnight. Sections were then incubated with Alexa Fluor 488-labeled donkey anti-IgG secondary antibody (Molecular Probes Inc) for 90 minutes at room temperature. After a rinse in Tris-buffered saline, sections were mounted with Vectashield (Vector Laboratories), and the staining was visualized with a microscope (LSM-510; Carl Zeiss AG). The number of Mac-2 cells on the dorsal surface of the brain was counted per 10 sagittal slices.

To visualize the sections of leptomeningeal arteries, other animals were used for immunostaining with α-smooth muscle actin (α-SMA) monoclonal antibody (1:400; Laboratory Vision). Mice were killed with carbon dioxide, and the brain was removed carefully and immersed in methanol followed by embedded in paraffin. Sagittal 5-μm-thick sections were immunostained with α-SMA using the Vector M.O.M. Immunodetection Kit (Vector Laboratories) and visualized with a microscope (TE2000-U; Nikon).

Statistical Analysis
All data are expressed as mean±SD. The Mann-Witney U test and Student t test were used for comparison of values between groups. Statistical significance was set at P<0.05.

Results
Decrease in Cerebral Perfusion Over the ACA and MCA Areas After Ipsilateral CCA Occlusion
Relative values of cortical perfusion over the ACA and MCA areas after ipsilateral CCA occlusion were 93.0±8.3% of baseline (n=9) and 56.8±3.7% of baseline (n=9), respectively. The decrease in perfusion was smaller in the ACA area than in the MCA area (P<0.01). This results suggested that CCA occlusion induces a perfusion pressure gradient between the ACA and MCA areas.

Infarct Volume Attributable to Permanent MCA Occlusion After CCA Occlusion
There was no significant difference in infarct size after MCA occlusion between the sham group (50.5±12.8 mm³, n=6) and the 7 days CCA occlusion group (46.7±13.1 mm³, n=6). However, infarct size attributable to MCA occlusion 14 days after CCA occlusion was smaller than that without CCA occlusion (25.5±7.8 mm³, n=6, versus 50.5±12.8 mm³, n=6, respectively, P<0.01; Figure 1).
Vessel Diameters of Leptomeningeal Anastomosis After CCA Oclusion
We hypothesized that a perfusion pressure gradient between the MCA and ACA areas after CCA occlusion induces the leptomeningeal collateral growth. The diameter of leptomeningeal collateral vessel 14 days after CCA occlusion (31.0±9.4 μm, n=4, 64 vessels) was larger than that in the sham group (25.6±4.5 μm, n=4, 64 vessels) or in the 7 days CCA occlusion group (25.9±7.7 μm, n=4, 65 vessels; Figure 2). Leptomeningeal collateral growth was present 14 days after CCA occlusion, whereas significant collateral growth was not identified 7 days after CCA occlusion.

Vessel Diameters After CCA Occlusion With or Without GM-CSF Treatment
We hypothesized that some arteriogenic factor, such as GM-CSF, may enhance collateral growth after CCA occlusion. There was no significant difference in diameter of leptomeningeal collateral vessel between the sham-saline group (24.4±4.5 μm, n=8, 136 vessels) and the sham-GM-
CSF group (25.4±5.8 μm, n=8, 130 vessels). However, the diameter of leptomeningeal anastomosis in the CCA-GM-CSF group (32.0±8.8 μm, n=8, 127 vessels) was markedly larger than that in the CCA-saline group (25.9±8.1 μm, n=8, 126 vessels, P<0.01) (Figure 3A, 3C, 3E). Thus, in the absence of a preceding chronic mild decrease in cerebral perfusion, GM-CSF treatment did not enhance leptomeningeal collateral growth. However, leptomeningeal collateral growth was markedly enhanced by GM-CSF treatment 7 days after CCA occlusion. The number of leptomeningeal collaterals did not increase after CCA occlusion or GM-CSF treatment (data not shown).

There was no significant difference in the diameter of the circle of Willis between the sham-saline group (140±13 μm, n=8) and the sham-GM-CSF group (131±11 μm, n=8). The diameter of the circle of Willis after CCA occlusion was larger in the CCA-saline group (189±58 μm, n=8) and CCA-GM-CSF group (215±12 μm, n=8) than in the sham-saline and the sham-GM-CSF groups (P<0.01). There was no significant difference in the diameter of the circle of Willis between the CCA-saline and CCA-GMCSF groups (Figure 3B and 3D). Collateral growth in the circle of Willis was present after unilateral CCA occlusion, but it was not enhanced by GM-CSF treatment.

Mac-2⁺ Cells on the Dorsal Surface of the Brain After CCA Occlusion and GM-CSF Treatment
The number of Mac-2⁺ cells on the dorsal surface of the brain in the CCA-saline group (68.4±23.3, n=8) was greater than that in the sham-saline group (37.3±26.2, n=8, P<0.05), and that in CCA-GM-CSF group (114.9±50.9, n=8) was greater than that in the CCA-saline group (68.4±23.3, n=8, P<0.05) and the sham-GM-CSF group (64.5±26.6, n=8, P<0.05; Figure 4). GM-CSF treatment enhanced the increase in monocyte/macrophage number on dorsal surface of the brain after CCA occlusion.

Infarct Volume Attributable to Permanent MCA Occlusion After CCA Occlusion With or Without GM-CSF Treatment
There was no significant difference in infarct volume attributable to MCA occlusion between the sham-saline and sham-GM-CSF groups (51.9±11.1 mm³, n=8, versus 52.0±15.0 mm³, n=8). Seven days after CCA occlusion, infarct size attributable to MCA occlusion in the CCA-GM-CSF group was smaller than that in the CCA-saline group (35.4±12.2 mm³, n=9 versus 48.0±14.8 mm³, n=9, P<0.05; Figure 5). Thus, GM-CSF treatment decreased the infarct volume attributable to MCA occlusion 7 days after CCA occlusion. However, in the absence of a preceding chronic mild decrease in cerebral perfusion by unilateral CCA occlusion, GM-CSF treatment did not decrease the infarct volume after permanent MCA occlusion.

Discussion
The present study showed that (1) unilateral CCA occlusion induced leptomeningeal collateral growth as well as enlargement of the circle of Willis in mice, (2) leptomeningeal collateral growth after unilateral CCA occlusion was enhanced by GM-CSF treatment, (3) monocyte/macrophage lineage was associated with unilateral CCA occlusion, and (4) after CCA occlusion, GM-CSF treatment decreased the infarct volume attributable to MCA occlusion.

Busch et al reported that CBF after ipsilateral CCA occlusion without vertebral artery occlusion decreased to 55% of the baseline value and returned to baseline within a few minutes in Sprague-Dawley rats. Only after 3-vessel occlusion, CBF remained at approximately 50% and collateral growth at the circle of Willis occurred.¹ However, in the present study, CBF over the MCA area after ipsilateral CCA occlusion remained at 56.7% of the baseline value in C57BL/6 mice. We also found that unilateral CCA occlusion in mice with a sustained decrease in CBF showed collateral growth at the circle of Willis.

Coyle⁰ reported that rapid occlusion of the MCA in rats resulted in dorsal collateral growth. However, it is well known that permanent MCA occlusion leads to brain infarction. To the best of our knowledge, there is not adequate experimental model in which chronic mild hypoperfusion without infarction is induced and leptomeningeal collateral growth can be investigated. In the present study, we found...
Fluid shear stress (FSS) is thought to be the initiating force in arteriogenesis. Pipp et al \textsuperscript{12} reported that increased FSS after femoral artery occlusion, plus arteriovenous shunting between the distal stump of the occluded artery and the accompanying vein, enhanced postocclusive arteriogenesis. Increased blood flow through leptomeningeal anastomosis in this unilateral CCA occlusion model should increase FSS directly and enhance arteriogenesis. Mechanical force exerted against endothelial cells is transmitted from the cell membrane to the nucleus, which activates transcription factors that switch on gene expression of chemokines such as MCP-1 and adhesion molecules such as ICAM-1, which are necessary for the docking of monocyte. \textsuperscript{11} Monocytes/macrophages accumulate around collateral vessels after arterial occlusion by ICAM-1/Mac-1–dependent mechanisms. \textsuperscript{13} Local infusion of MCP-1 or GM-CSF results in significant collateral artery growth after femoral artery occlusion, \textsuperscript{14,15} whereas a deficiency of circulating monocytes decreases collateral arterial growth. \textsuperscript{16} Buschmann et al \textsuperscript{4} reported that monocyte/macrophage lineage is associated with collateral growth and that GM-CSF enhances the collateral growth in the rat brain. GM-CSF is well known to increase macrophage survival. \textsuperscript{17} GM-CSF is produced and released by monocytes, macrophages, and endothelial cells, and its receptor has been detected on the surface of endothelial cells. \textsuperscript{18} GM-CSF produced by endothelial cells act in an autocrine or paracrine manner to affect the adhesion of leukocytes to endothelial cells by modulating adhesion molecule expression on the surface of endothelial cells, \textsuperscript{19} and the GM-CSF production is enhanced by FSS. \textsuperscript{20} The results of the present study showed that monocyte/macrophage lineage was associated with leptomeningeal collateral growth and showed that GM-CSF enhanced the enlargement of leptomeningeal collateral vessels.

Recent studies have reported that GM-CSF enhances cerebral collateral growth in the rat brain. \textsuperscript{4,21} These studies showed that GM-CSF enhances collateral growth in the P1 segment of the PCA or the posterior communicating artery (PComA) but not in the A1 segment of the ACA or the supraclinoid internal carotid artery (ICA) within the circle of Willis. In the present study, GM-CSF did not enhance collateral growth in the ACA or ICA within the circle of Willis in mice. We could not assess collateral growth in the P1 segment of the PCA or the PComA because the P1 segment in mice is narrow, lacking, or variable. \textsuperscript{22} In a rat model of bilateral CCA occlusion with an interval of 1 week between each occlusion, 5 weeks of GM-CSF treatment increased the number of leptomeningeal collateral vessels joining the ACA to the MCA and intraparenchymal arterioles but did not enlarge the vessels. \textsuperscript{21} Bilateral CCA occlusion may decrease the cerebral perfusion not only over the MCA area but also over the ACA area, whereas unilateral CCA occlusion resulted in little decrease of cerebral perfusion over the ipsilateral ACA area in the present study. Thus, the mechanism of leptomeningeal collateral growth in unilateral CCA occlusion in mice may differ from that in bilateral CCA occlusion in rats.

We confirmed results of our previous study that a chronic mild decrease in cerebral perfusion induced by unilateral CCA occlusion resulted in attenuation in infarct size after MCA occlusion 14 days later. \textsuperscript{5} However, 7 days after CCA occlusion, the infarct size attributable to permanent MCA occlusion was not decreased, probably because leptomeningeal collateral growth (26.5±8.1 μm in diameter) was insufficient to compensate for the ischemia induced by MCA occlusion. GM-CSF treatment decreased the infarct size after permanent MCA occlusion in these mice, probably because further enlargement of leptomeningeal collateral vessels (32.0±8.8 μm in diameter) induced by GM-CSF treatment was sufficient to compensate for the ischemia induced by MCA occlusion.

![Figure 5. A. Representative TTC staining of the brain after MCA occlusion. Seven days after CCA occlusion, a smaller infarct area was observed in the GM-CSF–treated mice (CCA-GM-CSF). B. Infarct volume attributable to MCA occlusion after sham CCA surgery (Sham) and 7 days after CCA occlusion (CCA) with GM-CSF or saline treatment. GM-CSF treatment decreased the infarct volume attributable to MCA occlusion 7 days after CCA occlusion. *P<0.05 compared to the sham-GM-CSF and CCA-saline groups.](http://stroke.ahajournals.org/content/41/12/1880/F5.large.jpg)
In conclusion, we could report for the first time that after CCA occlusion, GM-CSF enhanced leptomeningeal collateral growth and decreased the infarct volume after subsequent MCA occlusion. These results are consistent with those of recent studies in which GM-CSF enhanced cerebral collateral growth in rat brain.4,21 Clinically, patients with major cerebral occlusive disease and misery perfusion have a high risk of recurrent ischemic stroke.23 Extracranial-intracranial bypass surgery is available for such patients. However, a previous study failed to prove that this bypass surgery reduces the risk of ischemic stroke.24 There is no currently available pharmacological treatment to enhance collateral artery growth and decrease the risk of stroke in patients with compromised CBF. The results of the present study suggest that GM-CSF treatment to induce leptomeningeal arteriogenesis and decrease the risk of brain infarction is a promising approach for patients with misery perfusion. Further experimental and clinical studies are needed to establish this pharmacological treatment in patients with major cerebral occlusive disease.

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Disclosures
None.

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