Granulocyte Colony-Stimulating Factor Enhances Arteriogenesis and Ameliorates Cerebral Damage in a Mouse Model of Ischemic Stroke

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Background and Purpose—Enhancing collateral artery growth is a potent therapeutic approach to treat cardiovascular ischemic disease from occlusive artery. Granulocyte–macrophage colony-stimulating factor (GM-CSF) has gained attention for its ability to promote arteriogenesis, ameliorating brain damage, by the mechanisms involving monocyte upregulation. However, the recent clinical study testing its efficacy in myocardial ischemia has raised the question about its safety. We tested alternative colony-stimulating factors for their effects on collateral artery growth and brain protection.

Methods—Brain hypoperfusion was produced by occluding the left common carotid artery in C57/BL6 mice. After the surgery, granulocyte colony-stimulating factor, macrophage colony-stimulating factor, or GM-CSF (100 µg/kg/day) was administered daily for 5 days. The angioarchitecture for leptomeningeal anastomoses and the circle of Willis were visualized after the colony-stimulating factor treatment. Circulating blood monocytes and Mac-2-positive cells in the dorsal surface of the brain were determined. A set of animals underwent subsequent ipsilateral middle cerebral artery occlusion and infarct volume was assessed.

Results—Granulocyte colony-stimulating factor as well as GM-CSF promoted leptomeningeal collateral growth after common carotid artery occlusion. Both granulocyte colony-stimulating factor and GM-CSF increased circulating blood monocytes and Mac-2-positive cells in the dorsal brain surface, suggesting the mechanisms coupled to monocyte upregulation might be shared. Infarct volume after middle cerebral artery occlusion was reduced by granulocyte colony-stimulating factor, similarly to GM-CSF. Macrophage colony-stimulating factor showed none of theses effects.

Conclusions—Granulocyte colony-stimulating factor enhances collateral artery growth and reduces infarct volume in a mouse model of brain ischemia, similarly to GM-CSF. (Stroke. 2011;42:770-775.)

Key Words: animal models ■ arteriogenesis ■ brain ischemia ■ colony-stimulating factors

The risk of ischemic stroke is greatly enhanced in a patient with reduced cerebral blood flow reserve.1 Collateral artery growth (arteriogenesis) plays a critical role in maintaining cerebral blood flow reserve after major artery occlusion and determines the severity of subsequent ischemic injury of the brain.2,3 Leptomeningeal anastomoses are the most important collateral pathways, especially after middle cerebral artery (MCA) occlusion, and are recruited when the anastomoses flow at the circle of Willis is not adequate.2,4 Therapeutic stimulation of arteriogenesis is an attractive approach for treatment of chronic brain ischemia given this natural adaptive process can be enhanced by exogenous application of certain cytokines.5

One such cytokine is granulocyte–macrophage colony-stimulating factor (GM-CSF) that stimulates growth and differentiation of hematopoietic cells and is clinically used to treat chemotherapy-induced neutropenia.6 The arteriogenic effect of GM-CSF was originally reported in heart and peripheral artery disease by mechanisms involved in mobilization of monocytes/macrophages. Buschmann et al for the first time provided evidence that the same scenario could be applied to cerebral ischemia as well.7 Experimental studies have shown that GM-CSF induces arteriogenesis in the hypoperfused brain of rats and mice, restores cerebral blood flow, and ameliorates severity of stroke.7–9 Therapeutic clinical application of GM-CSF was expected in both cardiovascular and cerebrovascular fields; however, recent clinical trial to test the efficacy of GM-CSF in coronary artery disease raised the critical safety issue and was prematurely terminated, in which 2 of the 7 patients given GM-CSF had acute coronary syndrome.10 Therefore, safer alternatives are awaited.

Granulocyte (G-CSF) and macrophage colony-stimulating factor (M-CSF) are categorized in the same class of hematopoietic growth factors as GM-CSF. We hypothesize that these...
colony-stimulating factors might exert arteriogenic effects given their potential to induce proliferation and differentiation of hematopoietic cells into white blood cells. G-CSF, in particular, has an advantage because it has been in clinical use for a long time and a comprehensive safety profile has been demonstrated. In the current study, we focused on and tested arteriogenic effects of colony-stimulating factors and their direct impact on stroke severity. We found that G-CSF, but not M-CSF, induces arteriogenesis and alleviates ischemic damage to the brain, similarly to GM-CSF.

Methods

Animals

Male C57BL/6 strain mice (12 to 16 weeks of age; Charles River Japan Inc) were used in this study. In the first experiment, G-CSF, M-CSF, GM-CSF, or vehicle was administered, respectively, after left common carotid artery (CCA) occlusion. G-CSF or vehicle was also administered after sham CCA operation. In the second experiment, G-CSF at the different concentration was administered, respectively, after CCA occlusion. The experimental procedures were approved by the Institutional Animal Care and Use Committee of the Osaka University Graduate School of Medicine, Japan.

Administration of Colony-Stimulating Factors

In the first experiment, recombinant human G-CSF (Kyowa-Kirin Inc, Yokohama, Japan), recombinant human M-CSF (Kyowa-Kirin Inc), or recombinant mouse GM-CSF (R&D Systems Inc; each at 100 μg/kg diluted in 0.1 mL saline) was administered subcutaneously for 5 consecutive days immediately after CCA occlusion. Vehicle-treated mice were administered 0.1 mL saline. In the second experiment, G-CSF at the different concentrations (1 μg/kg, 10 μg/kg, 100 μg/kg diluted in 0.1 mL saline) or vehicle (0.1 mL saline) was administered subcutaneously for 5 consecutive days immediately after CCA occlusion.

Visualization of Vessels by Latex Perfusion

After 7 days of CCA occlusion (n=5, each group), latex perfusion was performed to visualize the leptomeningeal anastomoses and the circle of Willis as reported before. The mice were anesthetized with 4.0% halothane, and the right atrium of the heart was incised for allowing venous outflow. The left ventricle of the heart was cannulated and injected at 150 mm Hg with 2 mL saline and subsequently 0.5 mL latex compound (Product No. 563; Chicago Latex Products Inc), which was mixed with 50 μL/mL carbon black (Bokusai; Fueki Inc) beforehand. Then, the brain was removed from the skull, and photographs of the dorsal and ventral brain surface were taken. The distal MCA was identified from its branch angle and subsequently at the point where the distal MCA joined the distal anterior cerebral artery or posterior cerebral artery. The vessel diameter of the leptomeningeal anastomoses was measured at the just proximal point where the left anterior cerebral artery diverges the olfactory artery.

Statistics

All data are presented as mean±SEM. Differences between multiple groups were compared by analysis of variance followed by Tukey multiple comparison test. Nonparametric data were analyzed with the Kruskal-Wallis test followed by the Mann-Whitney U test. A value of P<0.05 was considered statistically significant.

An expanded “Methods” section is provided in the online supplement (available at http://stroke.ahajournals.org).

Results

Enhanced Leptomeningeal Anastomoses Growth by G-CSF

The 5-day treatment effects of colony-stimulating factors (G-CSF, GM-CSF, and M-CSF) on leptomeningeal anastomoses development were assessed after 7 days of CCA occlusion by quantitative measurement of vessel diameters. G-CSF as well as GM-CSF significantly enlarged the vessel diameter of leptomeningeal anastomoses as compared with vehicle treatment (G-CSF, 27.5±1.3 μm, 61 vessels, GM-CSF, 28.0±1.8 μm, 59 vessels versus vehicle, 22.5±0.9 μm, 60 vessels, P<0.01, respectively; Figures 1A, 1C, and 1D). M-CSF failed to show such effects (22.8±0.8 μm, 59 vessels). The vessel diameter of the circle of Willis was enlarged after CCA occlusion (CCA occlusion, 189±6 μm, sham CCA operation, 127±8 μm, P<0.01) but was not altered by any colony-stimulating factor treatment (G-CSF, 201±8 μm, GM-CSF, 192±16 μm, M-CSF, 195±10 μm; Figures 1B and 1C).

Circulating Blood Monocytes and Tissue Macrophages

Previous reports have shown that monocyte/macrophage mobilization is essential for arteriogenesis. Circulating blood monocytes were markedly increased by G-CSF or GM-CSF treatment, but not by M-CSF (G-CSF, 547.8±114.1/μL, GM-CSF, 536.8±138.0/μL versus vehicle, 101.0±34.4/μL, P<0.05, respectively; M-CSF, 106.4±34.9/μL; Figure 2A). As expected, granulocytes and lymphocytes were also increased by G-CSF, but not by M-CSF or GM-CSF. Macrophages in the dorsal brain surface were identified with Mac-2 antibody and quantified (Figure 2B). The number of Mac-2-positive cells was significantly increased by G-CSF versus vehicle in the presence of preceding CCA occlusion. Similar results were obtained with GM-CSF treatment but not with M-CSF (G-CSF, 107.4±14.6, GM-CSF, 104.6±10.1 versus vehicle, 51.4±2.4, P<0.05, respectively; M-CSF, 65.1±7.7; Figure 2C). The increase in blood monocytes and tissue macrophage accumulation correlate well with the arteriogenic effects observed.

To further elucidate the mechanistic link between monocyte/macrophage mobilization and arteriogenesis, we tested the impact of monocyte depletion on arteriogenic effect by G-CSF. A single dose of 5-fluorouracil (5-FU) administration (150 mg/kg intraperitoneally) severely depleted blood monocytes at 4 days and 8 days after the injection (4 days, 13.0±3.6/μL and 8 days, 0/μL). This intervention significantly interfered with growth response of leptomeningeal anastomoses by G-CSF treatment (5-FU, 21.5±1.1 μm, 67 vessels versus vehicle, 27.5±1.9 μm, 69 vessels, P<0.01; Figure 2D).

Reduction of Infarct Volume by G-CSF After Subsequent MCA Occlusion

Next, neuroprotective effects were directly tested by assessing brain infarct volume after subsequent MCA occlusion (Figure 3A–B). G-CSF did not alter brain infarct volume in animals without preceding CCA occlusion (sham operation) but significantly reduced infarct volume versus vehicle in the presence of preceding CCA occlusion (G-CSF, 35.4±4.7 mm³ versus vehicle, 55.1±4.3 mm³, P<0.05), suggesting that the benefit might be attributable to collateral artery development by the treatment. Importantly, there was no significant difference in the neuroprotective impact between G-CSF and GM-CSF treatment (GM-CSF, 37.8±3.9 mm³). M-CSF again had no such effect (M-CSF,
49.0±3.7 mm³). We further assessed cerebral blood flow using laser Doppler flowmetry. Cortical perfusion after MCA occlusion was significantly improved by G-CSF or GM-CSF treatment in the presence of preceding CCA occlusion, consistent with infarct volume reduction (G-CSF, 36.8%±4.4%, GM-CSF, 37.7%±5.1% versus vehicle, 19.2%±1.8%, P<0.05, respectively; Figure 3C). We next tested involvement of angiogenesis in this process by examining capillary density in the peripheral MCA cortex after CCA occlusion. Capillary density was assessed by CD31 staining and was not altered by G-CSF treatment (G-CSF, 220.6±2.3 vessels/field versus vehicle, 226.2±7.9 vessels/field, nonsignificant; Figure 4A–B). These results suggest little contribution of the angiogenesis mechanism to cerebral protection in this model.

Dose-Dependent Response of G-CSF on Leptomeningeal Collateral Growth

Finally, we examined whether G-CSF stimulates collateral growth in a dose-dependent manner (Figure 5A). The vessel diameter of leptomeningeal anastomoses was assessed at 3 different doses of G-CSF treatment (1, 10, and 100 μg/kg) for 5 days after CCA occlusion. G-CSF significantly increased the vessel diameter at 10 and 100 μg/kg but failed to have effects at 1 μg/kg (10 μg/kg, 24.7±0.9 μm, 60 vessels, P<0.05, 100 μg/kg, 27.4±1.5 μm, 58 vessels, P<0.01 versus vehicle, 20.8±0.7 μm, 61 vessels, 1 μg/kg, 21.3±0.8 μm, 65 vessels). In correlation with the dose-dependent response on leptomeningeal collateral growth, G-CSF significantly reduced infarct volume after subsequent MCA occlusion at 10 and 100 μg/kg but failed to show effects at 1 μg/kg (10 μg/kg, 38.2±3.0 mm³, 100 μg/kg, 34.9±4.4 mm³, P<0.05, respectively, versus vehicle, 56.6±4.1 mm³, 1 μg/kg, 55.8±5.8 mm³; Figure 5B).

Discussion

The current study demonstrates that G-CSF, but not M-CSF, has collateral enhancing property in hypoperfused brain similar to GM-CSF, which leads to powerful neuroprotection against subsequent ischemic stroke. Although G-CSF was already reported to act as a direct neuroprotectant,14–17 the current study is the first to demonstrate arteriogenic action by G-CSF treatment in the brain. The previously reported mechanisms for neuroprotection include direct neuronal impact by stem cell mobilization, antiapoptosis, neurogenesis, and systemic anti-inflammatory effects.14,18,19 However, these mechanisms might contribute minimally to the alleviated cerebral damage after MCA occlusion in the current protocol, in which colony-stimulating factor treatment was terminated
3 days before creating stroke (by MCA occlusion) and plasma concentration of G-CSF returns to baseline within 48 hours of subcutaneous injection. Indeed, no infarct-reducing impact was observed in the G-CSF-treated group without preceding hypoperfusion, strongly indicating the major role for collateral artery growth (arteriogenesis) in neuroprotection observed in the current study.

Arteriogenesis is a process in which pre-existing collateral arterioles transform into functional collateral arteries. Although the precise molecular mechanisms for arteriogenesis remain poorly understood, studies have clarified the pivotal role for circulating cells, particularly monocytes, in this process. Monocytes adhere the shear stress-activated endothelium through intracellular adhesion molecule-1, migrate toward the intima and mature into macrophages, producing growth factors and proteases for collateral artery remodeling. Previous studies have shown that the amount of circulating blood monocytes positively correlates with arteriogenesis.
riogenesis promotion. Osteopetrotic mutant mice, lacking bone marrow macrophages or blood monocytes, show severely depressed arteriogenesis after femoral artery occlusion. On the other hand, 5-FU-induced impaired arteriogenesis is reversed by supplementation with isolated monocytes in a mice model of femoral artery occlusion. In the present study, enhanced arteriogenesis either by G-CSF or GM-CSF was associated with increase in circulating blood monocytes, and the arteriogenic effect of G-CSF was suppressed by monocyte depletion, suggesting G-CSF might share the same mechanism involved in monocyte mobilization. Intracellular adhesion molecule-1 upregulation by G-CSF was also reported in a mouse model of myocardial infarction, facilitating a monocyte migration process. The similar mechanism might be at play in our model, although we did not examine intracellular adhesion molecule-1 expression. Most importantly, however, we did observe an increase in macrophages (Mac-2-positive cells) in the dorsal surface of the hypoperfused brain by G-CSF as well as GM-CSF. These results suggest collateral remodeling might be achieved in a coordinated manner by mobilization of monocytes and their active migration to the tissue. G-CSF induces angiogenesis as well, a process in which new vessels arise from pre-existing ones. Lee et al reported that 3-day treatment of G-CSF (50 μg/kg/day) after MCA occlusion enhances angiogenesis in the rat brain. In the current study, however, the contribution of angiogenesis might be minimal because we failed to detect upregulation of capillary density. Although the reason for the discrepancy is unclear, it could be attributable to several differences used in the protocol (unilateral CCA occlusion versus MCA occlusion, dosage, duration) and/or species (mice versus rats).

Based on ample preclinical data showing the beneficial effects of G-CSF in stroke models, several clinical studies were initiated and some reported. We observed an arteriogenic effect of G-CSF at the dose of 10 μg/kg/day for 5 days. Recent clinical studies used G-CSF at 1 to 10 μg/kg for 5 days in patients with stroke and demonstrated its safety. Another trial is ongoing to test the safety at higher doses (up to 100 μg/kg/day or total of 180 μg/kg over 3 days). One particular safety concern might be the possibility that increased blood neutrophils aggregate within microvasculature and impair tissue circulation. However, Strecker et al revealed that leukocytosis by G-CSF is restricted to the vessel compartment and has no deleterious effect on ischemic brain in experimental animals. Furthermore, recent meta-analysis has shown that G-CSF, unlike GM-CSF, does not induce cardiovascular events in patients with myocardial infarction.

In summary, G-CSF enhances arteriogenesis similarly to GM-CSF in a mouse model of chronic brain hypoperfusion, providing powerful neuroprotection against stroke. Although the clinical application of GM-CSF was unsuccessful so far due to the safety issue, safe clinical application of G-CSF has been shown at lower doses. The arteriogenic action of G-CSF could be a potential target for patients with occlusive disease of a major cerebral artery.

Acknowledgments
We thank Ms C. Kurano and K. Nishiyama for secretarial assistance. M-CSF and G-CSF were provided by Kyowa-Kirin Inc, Tokyo, Japan.

Sources of Funding
This research was supported by a Grant-in-Aid from the Ministry of Education, Science, and Culture in Japan.
Disclosures

None.

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*Stroke*. 2011;42:770-775; originally published online January 21, 2011; doi: 10.1161/STROKEAHA.110.597799

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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Full Title

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Cover Title: G-CSF Enhances Arteriogenesis
Supplemental Methods

Common Carotid Artery Occlusion
General anesthesia was induced by 4.0% halothane and maintained at 1.0% halothane with an open facemask. The body temperature was monitored by a rectal thermometer and maintained at 37.0 ± 0.5°C using a heat lamp and mat. The left CCA was exposed and ligated by a silk suture. Sham CCA operation was performed in the same process without ligation.

Blood Analysis
After 4 days of CCA occlusion (n=5, each group), blood was obtained by puncturing the right ventricle of the heart. The hematology analyzer (SE-9000; Sysmex Inc) was used for total leukocyte counting (cells/μl). Microscopic differential leukocyte count was performed on May-Grunwald-Giemsa stained smears and expressed in the percentage of total leukocyte count. Absolute blood monocyte, granulocyte, and lymphocyte counts (cells/μl) were calculated from the total leukocyte count and their percentage of total leukocyte count.

Monocyte depletion
Monocyte depletion was achieved by a single intraperitoneal injection of 5-fluorouracil (5-FU) as reported previously. Before 4 days of CCA occlusion, 5-FU (150mg/kg diluted in 1.0ml saline) or vehicle (1.0ml saline) was administered. To verify the monocyte depletion, blood analysis was performed at 4 days and 8 days after the injection (n=5, each group).

Immunohistochemistry
After 7 days of CCA occlusion (n=7, each group), immunostaining was performed using Mac-2 antibody to assess the macrophages accumulation in the dorsal surface of the brain. Sagittal 50 μm-thick sections between 1.5 mm and 3.0 mm lateral from the midline were incubated with Mac-2 antibody (1:200; Cedarlane Laboratories), and then incubated with the Alexa Fluor 488 anti-rat IgG antibody (1:200, Molecular Probes). Ten sections were investigated and the total number of Mac-2 positive cells accumulating in the dorsal surface of the brain was counted.
After 7 days of CCA occlusion, the borderzone leptomeningeal arteries were immunostained using alpha-smooth muscle actin (α-SMA) antibody. Borderzone was
interpreted as the area between 1.5 mm and 3.0 mm lateral from the midline, based on the location of leptomeningeal anastomoses. Sagittal 5μm-thick paraffin sections were immunostained with α-SMA antibody (1:400, Laboratory Vision) using the Vector M.O.M. Immunodetection Kit (Vector Laboratories).

After 7 days of CCA occlusion (n=5, each group), capillary density was assessed by CD31 staining as reported previously. Coronal 14μm-thick sections between 0.5mm and 1.0mm rostral to the bregma were incubated with CD31 antibody (1:50, BD Bioscience), and then incubated with the Biotinylated anti-rat IgG antibody (1:200, Vector Laboratories). The number of CD31 positive vessels in the peripheral MCA cortex was counted in the same manner through a ×20 objective lens. Capillary density (vessels/field) was calculated as the mean of the vascular counts obtained from three pictures. The stained sections were visualized with a microscope (Eclipse 80i; Nikon Inc).

Middle Cerebral Artery Occlusion Subsequent to Common Carotid Artery Occlusion

After 7 days of CCA occlusion (n=6, each group), the MCA was occluded using electrocoagulation. Under general anesthesia, the mice were placed in the recumbent position, and the skin was incised vertically at the midpoint between the left orbit and the external auditory canal. The mandible was pulled downward and then the left MCA was visualized through the skull. A small burr hole was made in the skull using a dental drill. The MCA was occluded permanently by a microbipolar electrocoagulator at the just proximal point where the olfactory branch came off. The body temperature was monitored by a rectal thermometer and maintained at 37.0±0.5°C using a heat lamp and mat. Twenty-four hours after MCA occlusion, the brain was removed for evaluation of infarct volume. The infarct volume was evaluated in 8 coronal sections of 1 mm-thickness stained with 2,3,5-triphenyltetrazolium-chloride/saline (TTC). The infarct area was measured with NIH Image V. 1.61 software, and the infarct volume was determined by integrating the infarct area of the 8 sections.

Measurement of Cortical Perfusion by Laser-Doppler Flowmetry

Under general anesthesia, a polyacrylamide column with an inner diameter of 0.8mm was attached to the skull 3.5mm lateral to the bregma with a dental cement, for measurement of cortical perfusion by laser-Doppler flowmetry (Advance laser Flowmetry, model ALF-21; Advance Co) as reported before. Cortical perfusion was measured before CCA occlusion (baseline), and again after MCA occlusion (n=5, each
Cortical perfusion after MCA occlusion was expressed as the percentage of the baseline cortical perfusion.

**Supplemental References**


Abstract

顆粒球コロニー刺激因子は虚血性脳卒中マウスモデルの動脈新生を促進し、脳損傷を軽減する

Granulocyte Colony-Stimulating Factor Enhances Arteriogenesis and Ameliorates Cerebral Damage in a Mouse Model of Ischemic Stroke

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背景および目的：側動脈動作の発現促進は、動脈閉塞による虚血性脳梗塞に対する有効な治療アプローチである。顆粒球コロニー刺激因子（GM-CSF）は、単球の増加を含む機序により、動脈新生を促進し、脳損傷を軽減する効果があることが指摘されている。一方、心筋虚血に対する有効性を確認した最近の臨床試験では、その安全性に疑問が投げかけられている。本研究では、GM-CSF

治療の脳梗塞に対する効果を用いて、側動脈閉塞の発現および脳保護に対する作用を検討した。

方法：CSF/BL5マウスの脳梗塞モデル（CCA）を選択し、脳梗塞を作成した。脳梗塞後、顆粒球コロニー刺激因子、マクロファージコロニー刺激因子、またはGM-CSF（100 μg/kg/day）を5日間与えて腫脹抑制を観察した。GM-CSF投与の腫脹抑制効果を検証した。

結果：脳梗塞マウスモデルにおいて、顆粒球コロニー刺激因子およびGM-CSFの両者は虚血性脳梗塞の単球と脳死脳のMacs-2陽性細胞を増加させ、共通して単球の増加をもたらすこれらの機序が関与していることが示された。顆粒球コロニー刺激因子を投与した場合では、MCA閉塞後の脳保護が、GM-CSFを投与した場合と同様に減少した。マクロファージコロニー刺激因子にこうした作用はみられなかった。

結論：脳梗塞実験において、顆粒球コロニー刺激因子およびGM-CSFは脳梗塞の発現を促進し、脳保護を減少させる。

Stroke 2011; 42: 770-775

図3

A: G-CSF投与により、MCA閉塞後の脳梗塞体積がGM-CSFと同様に減少した。
B: 2,3,5-トリフェニルチヤラゾリンウロブラクトクライン溶

液で作製したMCA閉塞後の脳梗塞体積が、GM-CSFまたは侵襲を投与し、7日後にMCAを開放した。C: CCA閉塞後の脳梗塞体積が、GM-CSF、2,3,5-トリフェニルチヤラゾリ

ンウロブラクトクライン溶液で作製したMCA開塞に対する効果を検討した。

GCSCF: 顆粒球コロニー刺激因子、GM-CSF: 顆粒球マクロファージコロニー刺激因子、M-CSF: マクロファージコロニー刺激因子、CCA: 細動脈閉塞。