Chronic Elevation of Tumor Necrosis Factor-α Mediates the Impairment of Leptomeningeal Arteriogenesis in db/db Mice

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Background and Purpose—Leptomeningeal collateral growth is a key factor that defines the severity of ischemic stroke. Patients with stroke generally have vascular risk factors, such as diabetes mellitus; however, consensus is lacking on how diabetes mellitus affects leptomeningeal arteriogenesis. We investigate the influence of diabetes mellitus on the leptomeningeal arteriogenesis.

Methods—We measured the vessel diameter of the leptomeningeal anastomoses 14 days after the common carotid artery occlusion in db/db, db/+ mice, and streptozotocin-induced hyperglycemic mice. In another set of these mice, we measured the infarct volume attributed to subsequent middle cerebral artery occlusion 14 days after the common carotid artery occlusion. Mac-2–positive cells on the dorsal brain surface and the mRNA expression of several macrophage-related factors in the cerebral cortex were examined. Finally, we tested whether the leptomeningeal arteriogenesis could be restored by pharmaceutical intervention in the db/db mice.

Results—Cerebral hypoperfusion led to significant ipsilateral leptomeningeal collateral growth in db/+ mice and streptozotocin-induced hyperglycemic mice. The collateral growth contributed to reduced infarct volume. In contrast, leptomeningeal arteriogenesis was impaired in the db/db mice. The number of Mac-2–positive cells was increased and tumor necrosis factor-α mRNA expression was induced after common carotid artery occlusion in the db/+ mice. However, these responses were not observed in the db/db mice. Administration of the tumor necrosis factor-α inhibitor etanercept before common carotid artery occlusion restored the hypoperfusion-induced leptomeningeal collateral growth in db/db mice.

Conclusions—These results indicate that leptomeningeal arteriogenesis is impaired in db/db mice and that suppression of the tumor necrosis factor-α response to hypoperfusion is the major contributing factor. (Stroke. 2015;46:1657-1663. DOI: 10.1161/STROKEAHA.114.008062.)

Key Words: animal models ■ collateral circulation ■ diabetes mellitus ■ tumor necrosis factor-α

Collateral arterial growth (arteriogenesis) plays a crucial role in ischemic stroke and is a key factor that defines the severity and functional prognosis of this disease. The circle of Willis and leptomeningeal anastomoses are typical intracranial collaterals. In the case of middle cerebral artery occlusion, the Circle of Willis is no longer able to contribute collateral blood supply and leptomeningeal anastomoses become the primary source of collateral blood flow. We previously reported that unilateral common carotid artery (CCA) occlusion induces ipsilateral leptomeningeal collateral growth after 14 days in rodents. Using this established model to estimate leptomeningeal arteriogenesis, we showed that hematopoietic factors enhance arteriogenesis in mice. We also demonstrated that leptomeningeal collateral growth induced by cerebral hypoperfusion is impaired in spontaneous hypertensive rats and apolipoprotein E–knockout mice. Vascular risk factors induce vascular dysfunction and may be related to impaired arteriogenesis. Diabetes mellitus is also a major vascular risk factor because the prevalence of hypertension, dyslipidemia, and diabetes mellitus is increasing worldwide. Diabetes mellitus is associated with an increased risk of first ischemic stroke, and 60% to 70% patients with ischemic stroke have diabetes mellitus or prediabetes mellitus. It was previously reported that diabetes mellitus impairs arteriogenesis in peripheral and coronary arteries, but it is not fully understood whether it impairs arteriogenesis in the cerebral vessels.
Because it was previously reported that monocytes and macrophages play an essential role in arteriogenesis, we searched the profiles of several macrophage-related factors after CCA occlusion. During the screening process, we focused on the tumor necrosis factor-α (TNF-α), a proinflammatory cytokine that plays important roles in inflammation, cellular proliferation, apoptosis, and morphogenesis.10 TNF-α is primarily secreted by monocytes and macrophages and is related to various pathological conditions, such as cancer, cardiovascular diseases, and metabolic diseases. TNF-α mediates the impairment of vascular function associated with the insulin resistance and diabetes mellitus that accompany obesity. It was also previously reported that TNF-α plays an important role in arteriogenesis.12–14

In this study, we hypothesized that TNF-α signaling perturbations might be related to impaired collateral growth in diabetes mellitus. Here, we investigated the leptomeningeal arteriogenesis induced by cerebral hypoperfusion in db/+ mice and found that abnormal TNF-α signaling was associated with impaired leptomeningeal arteriogenesis. In addition, we tested the effect of TNF-α inhibition before CCA occlusion on collateral growth.

Materials and Methods

More details are available in the online-only Data Supplement.

Animals

Db/db (BKS.Cg-Dock7+/Leprdb/J; type 2 diabetes mellitus) and db/+ (normoglycemic genetic controls of db/db) mice (12–13 weeks old; Charles River Japan Inc) were used in this study. We also used C57BL/6 strain mice (11–12 weeks old) intraperitoneally injected with streptozotocin (250 mg/kg). All mice were given free access to food and water before and after all procedures. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the Osaka University Graduate School of Medicine and all experiments were performed in accordance with the Osaka University Regulations on Animal Experiments. We assigned the db/db mice, db/+ mice, and streptozotocin-induced hyperglycemic mice to the CCA occlusion or sham groups. Latex perfusion was performed to visualize the leptomeningeal anastomoses 14 days after CCA surgery, as previously reported.1 In another set of mice, the left middle cerebral artery was occluded by electrocoagulation 14 days after the CCA surgery as previously described.15

Etanercept Administration

In the experiment with the TNF-α inhibitor etanercept, the db/db mice were injected intraperitoneally with either etanercept (5 mg/kg per day) or saline 3× daily on alternate days. Detailed protocol is available in the online-only Data Supplement methods.

Results

Hypoperfusion-Induced Leptomeningeal Collateral Growth Was Impaired in db/db Mice

The mean body weight, blood glucose level, and decrease of cerebral blood flow after CCA occlusion were shown in Figure I in the online-only Data Supplement. The same degree of cerebral blood flow reduction was observed after CCA occlusion in both db/+ and db/db mice. In the db/+ mice, there were significant increases in the leptomeningeal collateral vessel diameters in response to CCA occlusion (CCA occlusion, 33.7±1.2 μm, n=5 versus sham, 26.5±1.0 μm, n=5; P<0.05; Figure 1A and 1B) and infarct size was significantly attenuated, especially in the cerebral cortex (Figure II in the online-only Data Supplement), in the CCA occlusion group compared with the sham group (CCA occlusion, 21.2±0.9 mm³, n=7 versus sham, 38.3±3.4 mm³, n=7; P<0.05; Figure 1C and 1D), which was in agreement with our previous study in normal rodents. In contrast, in the db/db mice, there were no significant differences in the vessel diameters between the CCA occlusion group and the sham group (CCA occlusion, 27.9±1.0 μm, n=5 versus sham, 26.6±1.0 μm, n=5; Figure 1A and 1B) and no reductive effect of infarct size attributed to leptomeningeal arteriogenesis (CCA occlusion, 34.0±4.1 mm³, n=7 versus sham, 35.7±1.7 mm³, n=7; Figure 1C and 1D).

Figure 1. Leptomeningeal collateral growth by common carotid artery (CCA) occlusion was impaired and no reductive effect was observed after subsequent middle cerebral artery (MCA) occlusion in db/db mice. A, Representative images of leptomeningeal anastomoses after CCA occlusion in db/+ and db/db mice. Scale bar, 100 μm. B, Vessel diameter of leptomeningeal anastomoses after CCA occlusion in db/+ and db/db mice (n=5). Leptomeningeal collateral growth was impaired in db/db mice. *P<0.05 compared with the sham group. C, Representative 2,3,5-triphenyltetrazolium-chloride/saline staining of the brain after MCA occlusion. MCA occlusion was performed 14 days after sham CCA occlusion in db/+ and db/db mice. D, Infarct volume attributable to MCA occlusion 14 days after CCA occlusion in db/+ and db/db mice. In contrast to db/+ mice, reduced infarct volume was not observed in db/db mice. *P<0.05 compared with the sham group. CCAO indicates common carotid artery occlusion.
Leptomeningeal Arteriogenesis Was Not Impaired in Streptozotocin-Induced Hyperglycemic Mice

To investigate the influence of hyperglycemia on leptomeningeal arteriogenesis, we used the streptozotocin-induced hyperglycemic mice. The mean body weight and blood glucose level were shown in Figure III in the online-only Data Supplement. The blood glucose levels of the streptozotocin-induced hyperglycemic mice were comparable to those of the db/db mice. In contrast to the db/db mice, there were significant increases in the leptomeningeal collateral vessel diameters (CCA occlusion, 29.1±1.1 μm versus sham, 23.6±0.7 μm; P<0.05; Figure 2A and 2B) and significantly reduced infarct size in response to CCA occlusion (CCA occlusion, 28.7±2.1 mm³, n=7 versus sham, 40.3±1.9 mm³, n=7; P<0.05; Figure 2C and 2D) in streptozotocin-induced hyperglycemic mice. This finding suggests that hyperglycemia during cerebral hypoperfusion was not the main cause of impaired arteriogenesis in the db/db mouse.

Numbers of Macrophages on the Dorsal Surface of the Brain Were Significantly Increased 7 Days After CCA Occlusion in the db/+ Mice But Not in db/db Mice

We previously demonstrated that the number of macrophages on the dorsal brain surface was significantly increased 7 days after CCA occlusion.5 In the db/+ mice examined here, the number of Mac-2–positive cells was significantly increased 7 days after CCA occlusion (CCA occlusion, 103±7, n=6 versus sham, 76±8, n=6; P<0.05; Figure 3A and 3B). However, the db/db mice in the sham group had larger numbers of Mac-2–positive cells than the db/+ mice in the sham group. However, no reactive increase of the number of Mac-2–positive cells was observed in the db/db mice 7 days after CCA occlusion (CCA occlusion, 138±15, n=6 versus sham, 124±9, n=6; Figure 3A and 3B).

TNF-α mRNA Expression in the Cerebral Cortex Was Significantly Increased 7 Days After CCA Occlusion in db/+ Mice But Not in db/db Mice

The result of Mac-2 staining suggested that the difference in macrophage mobilization between db/+ and db/db mice might contribute to the impaired leptomeningeal collateral growth in db/db mice. Next, we investigated the mRNA expression of several macrophage-related factors in the left cerebral cortex, including leptomeningeal anastomoses in db/+ and db/db mice using real-time polymerase chain reaction. Baseline mRNA expression of nitric oxide synthase 2, TNF-α, and arginase-1 was significantly increased in the db/+ mice compared with the db/+ mice (Figure 4A). When viewed from the perspective of changes over time, expression of some factors increased 7 days after CCA occlusion in db/+ mice. In particular, expression of TNF-α levels was significantly increased in db/+ mice (Figure 4B), but not in db/db mice (Figure 4C). TNF-α exerts its biological effects by interacting with 2 different TNF-α receptors (TNFR1 and TNFR2). TNFR1 and TNFR2 levels were significantly higher in the db/+ mice than in the db/+ mice (Figure 5A) and were significantly increased 7 days after CCA occlusion in the db/+ mice (Figure 5B). No significant increases were observed in the db/db mouse, as with TNF-α (Figure 5C).

Preadministration of Etanercept Restored Leptomeningeal Collateral Growth in the db/db Mice

The results to date suggest the possibility that chronic TNF-α elevation impaired the TNF-α signaling response in the brains of the db/db mice. TNF-α protein expression was also increased in the cerebral cortex of db/db mice compared with db/+ mice (Figure IV in the online-only Data Supplement).
We hypothesized that the leptomeningeal collateral growth induced by chronic hypoperfusion could be restored by TNF-α level normalization before CCA occlusion in the db/db mice (Figure 6A). We used the TNF-α inhibitor etanercept to reduce the excess TNF-α signaling. There were no significant differences in the body weights or blood glucose levels before versus after etanercept administration (Figure V in the online-only Data Supplement). The same degree of cerebral blood flow reduction was observed after CCA occlusion in db/db mice both with preadministration of etanercept and vehicle (Figure V in the online-only Data Supplement). The mean vessel diameter of the leptomeningeal anastomoses was significantly increased in the CCA occlusion with preadministration of etanercept group compared with the other groups (CCA occlusion with preadministration of etanercept, 30.1±1.0 μm, n=6, versus CCA occlusion with vehicle, 26.3±0.8 μm, n=6, versus sham with preadministration of etanercept, 24.4±0.6 μm, n=6; P<0.05; Figure 6B). In another set of mice, infarct size was significantly attenuated, especially in the cerebral cortex (Figure V in the online-only Data Supplement), in the CCA occlusion with preadministration of etanercept group compared with the CCA occlusion with vehicle group (vehicle group, 38.0±1.4 mm³, n=8 versus etanercept group, 29.3±3.3 mm³, n=8; P<0.05; Figure 6C). Neurological functional deficits were significantly attenuated in the CCA occlusion with preadministration of etanercept group compared with the CCA occlusion with vehicle group on modified neurological severity score (n=8 per group; Figure 6D; Table I in the online-only Data Supplement).

**Discussion**

In this article, we found that (1) leptomeningeal collateral growth induced by cerebral hypoperfusion after unilateral CCA occlusion is impaired in db/db mice; (2) hyperglycemia during cerebral hypoperfusion is not the main cause of impaired arteriogenesis in db/db mice; (3) no reactive increase of macrophages for unilateral CCA occlusion on the dorsal surface of the brain is observed in db/db mice; (4) mRNA expression of TNF-α was increased most significantly 7 days after CCA occlusion in db/+ mice, whereas there was no significant change over time in db/db mice; and (5) the administration of etanercept before unilateral CCA occlusion restored leptomeningeal collateral growth in db/db mice. To our knowledge, this is the first report to demonstrate that cerebral arteriogenesis for chronic hypoperfusion is impaired by diabetes mellitus. Furthermore, we found that TNF-α is strongly associated with the mechanism for cerebral arteriogenesis impairment and that the leptomeningeal collateral growth in db/db mice can be restored by intervention of the drug that has been already used in clinical practice.

Unlike angiogenesis, arteriogenesis occurs in response to increased fluid shear stress, and it exhibits the remodeling and growth of collateral arterioles from preexisting anastomoses. Angiogenesis results in an overall increment of vessel resistance and is insufficient to improve tissue perfusion; hence, arteriogenesis is a crucial natural compensatory mechanism for improving tissue perfusion for occlusive arterial disease.

We previously described that hypertension and hypercholesterolemia impaired hypoperfusion-induced collateral growth in the cerebral circulation. In this study, we demonstrated that leptomeningeal arteriogenesis was also impaired in db/db mice. Circrovascular density and collateralization were reportedly increased in Goto–Kakizaki (GK) rats of a mild and lean model of diabetes mellitus. Different from db/db mice, the GK rats are a model of mild type 2 diabetes mellitus without confounding factors, such as obesity. That is, this study investigated the effects of mild hyperglycemia alone on native leptomeningeal collateral state without inducing hypoperfusion. Another study comparing the native cerebral neovascularization of GK rats and db/db mice showed that the GK rats had increased microvessel and macrovessel densities, whereas the db/db mice had increased microvasculature densities and decreased lumen diameters of the penetrating arterioles. Our results are consistent with those of previous studies in that hyperglycemia alone does not impair leptomeningeal arteriogenesis and that the arteriole diameters of the db/db mice were not increased unlike those of the GK rats.

Although the exact molecular mechanisms of arteriogenesis remain largely unclear, several studies and reviews have demonstrated that macrophages play a pivotal role in this process. The increased shear stress in the collateral vessels activates the endothelium, where circulating monocytes adhere via upregulated intracellular adhesion molecule-1, transmigrate into the perivascular tissue, and mature into macrophages, producing growth factors and cytokines for collateral artery growth. In this study, we observed a higher number of macrophages on the dorsal surface of the brain in the db/db mice than in the db/+ mice without CCA occlusion, and the number of macrophages in the db/db mice did not change after CCA occlusion, whereas that in the db/+ mice was significantly increased 7 days after CCA occlusion. We previously reported that the same situation was observed in spontaneously hypertensive rats. These results suggest...
that tissue macrophage preaccumulation because of chronic inflammation attributable to vascular risk factors inhibits further macrophage mobilization for collateral artery growth. We investigated mRNA expression of representative macrophage-related factors in the cerebral cortex including leptomeningeal anastomoses in db/+ and db/db mice to elucidate a

Figure 4. The mRNA expressions of tumor necrosis factor (TNF-α) and arginase-1 in the left cerebral cortex including leptomeningeal anastomoses were significantly increased 7 days after common carotid artery (CCA) occlusion in db/+ mice but not in db/db mice. A. The mRNA expressions of representative macrophage-related factors in the left cerebral cortex, including leptomeningeal anastomoses in db/+ and db/db mice without CCA occlusion (n=7). The mRNA expressions of nitric oxide synthase (NOS) 2, TNF-α, and arginase-1 were significantly increased in db/db mice. *P<0.05 compared with the db/+ mice. B. The time-dependent change of mRNA expression of representative macrophage-related factors in db/+ mice (n=7). No significant increase was observed compared with baseline of db/db mice. Figure 5. The mRNA expression of tumor necrosis factor-α receptor (TNFR) 1 and receptor (TNFR) 2 were also increased 7 days after common carotid artery (CCA) occlusion in db/+ mice but not in db/db mice. A. The mRNA expressions of TNFRI and TNFR2 in the left cerebral cortex, including the leptomeningeal anastomoses in db/+ and db/db mice without CCA occlusion (n=7). The mRNA expressions of both the receptors were significantly increased in db/db mice. *P<0.05 compared with db/+ mice. B. The time-dependent change of mRNA expressions of TNFRI and TNFR2 in the left cerebral cortex, including the leptomeningeal anastomoses in db/+ mice. Both the receptors were significantly increased 7 days after CCA occlusion. *P<0.05 compared with baseline of db/+ mice (day 0). C. The time-dependent changes of mRNA expressions of TNFRI and TNFR2 in the left cerebral cortex, including the leptomeningeal anastomoses in db/db mice. No significant increase was observed compared with baseline of db/db mice.
key factor of diabetic impairment of leptomeningeal collateral growth. In this study, some factors, especially TNF-α, showed an interesting dynamic state of transient increases 7 days after CCA occlusion in db/+ mice, but not in db/db mice.

TNF-α is closely related to diabetes mellitus. TNF-α plays a pivotal role in mediating insulin resistance as a result of obesity.22,23 One experimental study showed that plasma concentrations of TNF-α were significantly increased in db/db mice and that TNF-α induced endothelial dysfunction in db/db mice.24 In terms of arteriogenesis, earlier studies suggested that TNF-α is expressed by macrophages around growing collateral arteries25 and that TNF-α functions as a positive modulator for arteriogenesis related to its activation of TNFR1 or TNFR2.12–14 In the process of arteriogenesis, TNF-α induces adhesion factors by sustaining inflammation and increasing monocyte and macrophage recruitment. Furthermore, TNF-α can activate nuclear factor-κB, and 1 report demonstrated that endothelial nuclear factor-κB signaling plays a key role in the regulation of arteriogenesis and the formation of the collateral circulation.26 Accordingly, TNF-α is closely involved with diabetes mellitus and arteriogenesis. In our results, CCA occlusion could not stimulate TNF-α signaling in db/db mice. One mechanism of such an abnormal response might be a resistant TNF-α state induced by chronically elevated TNF-α expression. Chronic increases in TNF-α stimulation to the cerebral vessels might induce resistance to TNF-α signaling in db/db mice.

To restore the leptomeningeal collateral growth in db/db mice by reducing TNF-α, we used etanercept, a soluble TNF-α receptor. Etanercept is widely used to treat rheumatoid arthritis and has an established clinical safety profile. Our result that leptomeningeal collateral growth was restored in db/db mice by etanercept preadministration strongly suggests that the impaired leptomeningeal collateral growth in db/db mice is mainly because of chronic exposure to TNF-α stimulation and downregulated vessel reactivity to the CCA occlusion stimulation.

Conclusions
This is the first report demonstrating that cerebral arteriogenesis for chronic hypoperfusion is impaired by diabetes mellitus. The impairment is not because of hyperglycemia alone but rather to chronic TNF-α stimulation to the cerebral vessels. Reducing TNF-α levels before inducing CCA occlusion can restore the leptomeningeal collateral growth in db/db mice. The data suggest that decreasing the responsiveness to TNF-α signaling is the major factor influencing diabetic impairment of cerebral arteriogenesis.

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Disclosures
None.
References


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SUPPLEMENTAL MATERIAL

Title
Chronic elevation of tumor necrosis factor (TNF)-α mediates the impairment of leptomeningeal arteriogenesis in type2 diabetic mice.

Cover title
TNF-α and impaired arteriogenesis in diabetes

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Supplemental Methods

CCA Occlusion
We assigned the db/db mice and db/+ mice used here to the left CCA occlusion or sham groups to examine the effect of diabetes on leptomeningeal collateral growth. The Streptozotocin (STZ) -induced hyperglycemic mice were also assigned to the two groups to investigate the influence of hyperglycemia.

General anesthesia was induced with 4.0% isoflurane and maintained at 1.5% isoflurane with an open facemask, and rectal temperature was monitored and maintained at 37 ± 0.5°C with a heat lamp and mat. In the occlusion group, the left CCA was exposed and ligated by a silk suture. In the sham group, the same operation was performed without ligation.

Measurement of Cerebral Blood Flow (CBF)
Changes in surface CBF were monitored using a laser speckle blood flow imaging system (Omegazone; Omegawave) that obtains high-resolution, two-dimensional images. The CBF recordings were obtained as described previously1,2,3 with some modifications. The mice were anesthetized with 1.5% isoflurane and placed in the prone position immediately after CCA occlusion. Rectal temperature was monitored and maintained at 37 ± 0.5°C with a heat lamp and mat. The skull was exposed by a midline scalp incision. The surface of the skull was moistened with saline to prevent drying. During the CBF measurement, the skull surface was diffusely illuminated by a 780-nm laser light. The scattered light was filtered and detected by a charge-coupled device camera positioned over the head. The filter detected only scattered light that had a perpendicular polarization to the incident laser light. The raw speckle images were used to calculate the speckle contrast. Signal processing was performed by the algorithm developed by Forrester et al. 4 Color-coded CBF images were obtained in high-resolution mode. The regions of interest corresponded with the regions around the leptomeningeal anastomoses connecting the dorsal branches of the anterior cerebral artery (ACA) and the MCA. Twenty CBF recordings were averaged and the CBF values were expressed as percentages of the contralateral (left/right) CBF values.

Streptozotocin (STZ) -induced hyperglycemic mice
C57BL/6 strain mice (11–12 weeks old) were injected with STZ (250mg/kg) intraperitoneally. One week after the STZ injection, mice with a blood glucose level > 450 mg/dL were chosen for the experiments.

Visualization of Leptomeningeal Anastomoses by Latex Perfusion
Under deep isoflurane anesthesia, rectal temperature was monitored and maintained at 37 ± 0.5°C with a heat lamp and mat. The right atrium of the heart was incised to enable venous outflow. The left ventricle of the heart was cannulated and saline (8 × 10−2 mL/mouse • g) was injected. Immediately after saline injection, latex compound (2 × 10−2 mL/mouse • g, Product No. 563; Chicago Latex Products Inc) mixed with 50 μL/mL carbon black (Bokusai; Fueki Inc) was injected at 150 mmHg. The brain was then removed from the skull and immersed in
Zamboni solution (2% paraformaldehyde and 0.2% picric acid) for 2 days. Photographs of the dorsal brain surface were then taken. The distal MCA was identified from its branch angle and distinguished from the distal ACA or posterior cerebral artery (PCA). The diameter of each leptomeningeal anastomosis was measured at the point of confluence between the distal MCA and the distal ACA or PCA in a blinded fashion.

**MCA Occlusion Subsequent to CCA Occlusion**

Under general anesthesia, the mice were placed in the recumbent position and the skin was incised longitudinally at the midpoint between the left orbit and the external auditory canal. The mandible was pulled downward and the left MCA was visualized through the skull. A small burr hole was made in the skull above the MCA using a dental drill. The MCA was occluded permanently by electrocoagulation just proximal to the point of olfactory branch origination. The rectal temperature was monitored and maintained at 37.0 ± 0.5°C using a heat lamp and mat. Operated mice were included if they showed spastic paralysis of the right forelimb after surgery. Twenty-four hours after the MCA occlusion, the brain was removed from the skull for infarct volume evaluation. The infarct volume was evaluated in eight 1-mm-thick coronal sections stained with 2,3,5-triphenyltetrazolium-chloride/saline (TTC). The infarct area was measured with Image J and the infarct volume was determined by integration of the infarct areas of the eight sections in a blinded fashion.

**Immunohistochemistry**

Immunostaining was performed using Mac-2 antibody to assess the degree of macrophage accumulation on the dorsal surface of the brain 7 days after CCA occlusion (n = 6 in each group). Sagittal 50-μm-thick sections taken 1.5–3.0 mm lateral of midline were incubated with Mac-2 antibody (1:200; Cedarlane Laboratories) at 4°C overnight and then incubated with Alexa Fluor 594 anti-rat immunoglobulin G antibody (1:200; Molecular Probes) at room temperature for 60 minutes. The total number of Mac-2–positive cells accumulating on the dorsal surface of the brain in 10 slices was counted.

**Real-Time Polymerase Chain Reaction (PCR)**

Total RNA was isolated from the left cerebral cortex including the leptomeningeal anastomoses using TRIZOL reagent (Life Technologies) according to manufacturer’s instructions, and then cDNA was synthesized using High-capacity cDNA Reverse Transcription Kits (Applied Biosystems). PCR was performed for several macrophage-related factors on a ViiA 7 real-time PCR system (Applied Biosystems). The cycle times were normalized to Rn18s of the same sample. The mRNA expression levels were reported as fold changes versus the sham group levels.

**Western Blot**

The brain tissue samples were taken from the left cerebral cortex including the leptomeningeal anastomoses. The samples were homogenized in buffer containing protease inhibitor cocktail (Complete Mini; Roche, Basel, Switzerland). Homogenates were then
centrifuged at 15,000 rpm for 30 min at 4°C. Brain homogenate supernatants were mixed with Laemmli sample buffer containing 2-mercaptoethanol and were boiled for 5 minutes. The protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and separated proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA, USA). Membranes were incubated with the following primary antibodies: mouse anti-TNFα antibody (abcam) and mouse anti-β-actin antibody (Sigma). Proteins were detected using the ECL detection system (GE Healthcare, Buckinghamshire, UK) after incubation with secondary antibodies (GE Healthcare). Signals were quantified using the Image J.

**Etanercept Administration**

In the experiment with the TNF-α inhibitor etanercept, we used only db/db mice (12–13 weeks old). The mice were divided into the following three groups: CCA occlusion group with pre-administration of vehicle (saline); CCA occlusion group with pre-administration of etanercept; and sham group with pre-administration of etanercept. The CCA surgery was performed 7 days after the first administration of etanercept or saline. Latex perfusion was performed 14 days after CCA surgery. Next, we performed the permanent MCA occlusion 14 days after CCA occlusion on different set of db/db mice divided into two groups (CCA occlusion group with pre-administration of vehicle (saline) and CCA occlusion group with pre-administration of etanercept). Twenty-four hours after the MCA occlusion, we checked the functional outcome and infarct volume.

**Functional Test**

In the experiment with the TNF-α inhibitor etanercept, a battery of behavioral test (modified Neurological Severity Score [mNSS]) was performed 24 hours after MCA occlusion by an investigator who was blinded to the experimental groups. Neurological function was graded on a scale of 0 to 14 (normal score 0; maximal deficit score 14, see supplemental Table I).

**Statistical Analysis**

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

**Supplemental References**


Supplemental Table I  Modified neurological severity score ( mNSS )

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<tr>
<th>Motor Tests</th>
<th>Points</th>
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<td><strong>Raising the mouse by tail</strong></td>
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<td>1. Flexion of forelimb</td>
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<td>1. Flexion of hindlimb</td>
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<td>1. Head moved more than $10^\circ$ to the vertical axis</td>
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<tr>
<td>0. Normal walk</td>
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<tr>
<td>1. Inability to walk straight</td>
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<td>2. Circling toward the paretic side</td>
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<tr>
<td>3. Falling down to the paretic side</td>
<td></td>
</tr>
<tr>
<td><strong>Baem balance tests ( normal = 0 ; maximum = 6 )</strong></td>
<td></td>
</tr>
<tr>
<td>0. Balances with steady posture</td>
<td></td>
</tr>
<tr>
<td>1. Grasps side of beam</td>
<td></td>
</tr>
<tr>
<td>2. Hugs the beam and one limb falls down from the beam</td>
<td></td>
</tr>
<tr>
<td>3. Hugs the beam and two limbs fall down from the beam, or spin on beam ( &gt;30 seconds )</td>
<td>6</td>
</tr>
<tr>
<td>4. Attempts to balance on the beam but fall off ( &gt;20 seconds )</td>
<td></td>
</tr>
<tr>
<td>5. Attempts to balance on the beam but fall off ( &gt;10 seconds )</td>
<td></td>
</tr>
<tr>
<td>6. Fall off: No attempt to balance or hang on to the beam( &lt; 10 seconds )</td>
<td></td>
</tr>
<tr>
<td><strong>Reflexes absence</strong></td>
<td></td>
</tr>
<tr>
<td>1. Pinna reflex ( a head shake when touching the auditory meatus )</td>
<td>2</td>
</tr>
<tr>
<td>1. Corneal reflex ( an eye blink when lightly touching the cornea with cotton )</td>
<td></td>
</tr>
<tr>
<td><strong>Maximum points</strong></td>
<td>14</td>
</tr>
</tbody>
</table>
Figure I. Characteristics of and cerebral blood flow (CBF) reductions after common carotid artery (CCA) occlusion in db/+ and db/db mice.

A, Body weights of the db/+ and db/db mice (N = 5). The mean body weight of the db/db mice was significantly heavier than that of the db/+ mice. 

B, Blood glucose levels of the db/+ and db/db mice (N = 5). The mean blood glucose level of the db/db mice was higher than that of the db/+ mice. 

C, Representative images of the CBF of the db/+ and db/db mice after CCA occlusion. 

D, The ratio of the CBF in the left middle cerebral artery (MCA) territory to that of the CBF in right MCA territory in db/+ and db/db mice after CCA occlusion (N = 5). The same degree of CBF reduction was observed after CCA occlusion in both db/+ and db/db mice.
Figure II. Leptomeningeal collateral growth in the surface of brain mainly contributed to reduce the infarct volume attributable to MCA occlusion 14 days after CCA occlusion in db/+ and streptozotocin (STZ) induced hyperglycemic mice.

A, Infarct volume attributable to MCA occlusion 14 days after CCA occlusion in the cortex and basal ganglia in db/+ mice (N = 7). Infarct size was significantly reduced in response to CCA occlusion in the cerebral cortex, not in the basal ganglia, in db/+ mice. *P < 0.05 compared to the sham group.

B, Infarct volume attributable to MCA occlusion 14 days after CCA occlusion in the cortex and basal ganglia in STZ-induced hyperglycemic mice (N = 7). Infarct size was significantly reduced in response to CCA occlusion in the cerebral cortex, not in the basal ganglia, in STZ-induced hyperglycemic mice. *P < 0.05 compared to the sham group.
Figure III. There were no differences in body weights or blood glucose levels between sham-operated and CCA-occluded mice.

A, Body weights of STZ-induced hyperglycemic mice (N = 5). B, Blood glucose levels of the STZ-induced hyperglycemic mice (N = 5).
Figure IV. Western blot analysis of TNF-α. TNF-α protein expression was significantly increased in the cerebral cortex of db/db mice. *P < 0.05 compared to the db/+ mice.
Figure V. Restored leptomeningeal collateral growth in the surface of brain mainly contributed to reduce the infarct volume attributable to MCA occlusion 14 days after CCA occlusion in db/db mice with pre-administration of etanercept.

A, Body weights and blood glucose levels of the db/db mice before and after etanercept administration (N = 6). There were no significant differences before versus after etanercept administration. B, Representative images of the CBF and the ratio of the CBF in the left middle cerebral artery (MCA) territory to that of the CBF in right MCA territory in db/db mice with etanercept or saline after CCA occlusion. (N = 5) The same degree of CBF
reduction was observed after CCA occlusion in both groups. C, Infarct volume attributable to MCA occlusion 14 days after CCA occlusion in the cortex and basal ganglia in db/db mice treated with etanercept or saline (N = 8). Infarct size was significantly attenuated in response to CCA occlusion in the cerebral cortex, not in the basal ganglia, in the CCA occlusion with pre-administration of etanercept group. *P < 0.05 compared to the CCA occlusion with vehicle group.
腫瘍壊死因子αの慢性的上昇は、db/dbマウスの脳軟膜の側
副血行発達不全の原因となる

Chronic Elevation of Tumor Necrosis Factor-α Mediates the Impairment of Leptomeningeal Arteriogenesis in db/db Mice

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Abstract

慢性の腫瘍壊死因子αの上昇は、db/dbマウスの脳軟膜の側
副血行発達不全の原因となる。db/dbマウスでは、脳皮質
の側副血行発達不全の原因となる。慢性の腫瘍壊死因子α
の上昇は、脳皮質の側副血行発達不全の原因となる。db/db
マウスでは、脳皮質の側副血行発達不全の原因となる。