Granulocyte-Macrophage Colony-Stimulating Factor–Induced Vessel Growth Restores Cerebral Blood Supply After Bilateral Carotid Artery Occlusion

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Background and Purpose—Hemodynamic compromise due to occlusive cerebrovascular disease is associated with an increased stroke risk. Granulocyte-macrophage colony-stimulating factor (GM-CSF) has been suggested to stimulate collateral blood vessel growth in various models of hemodynamic compromise. The purpose of this study was to investigate the effects of GM-CSF on cerebral hemodynamics and vessel growth in a rat model of chronically impaired cerebral blood flow (CBF).

Methods—Male Sprague-Dawley rats underwent sequential bilateral carotid artery occlusion (BCO) and were treated with GM-CSF or saline for 6 weeks. Sham-occluded animals served as a control group. Baseline CBF was measured by iodo[14C]antipyrine autoradiography, and cerebrovascular reserve capacity was assessed by laser-Doppler flowmetry after application of 20 mg/kg body weight acetazolamide. The capillary density and arterioles immunopositive for α-smooth muscle actin were counted on brain sections. The cerebral angiarchitecture was visualized with a latex perfusion technique.

Results—Baseline CBF as measured by iodo[14C]antipyrine autoradiography was not affected by BCO. The cerebrovascular reserve capacity, however, was significantly impaired 1 week after BCO. CBF and cerebrovascular reserve capacity recovered completely in GM-CSF–treated animals but not in solvent-treated animals. Histologic analysis of the hippocampus revealed integrity of the hypoxia-vulnerable neurons in all animals. The capillary density showed a very mild increase in GM-CSF–treated animals. However, the number of intraparenchymal and leptomeningeal arterioles was significantly higher in GM-CSF–treated animals than in both other groups.

Conclusions—Long-term GM-CSF treatment in a BCO model in rats leads to restoration of impaired cerebral hemodynamics and accompanies structural changes in the resistance-vessel network. (Stroke. 2007;38:1320-1328.)

Key Words: angiogenesis ■ cerebral blood flow ■ growth factors ■ stroke ■ vasculature

O cclusive cerebrovascular disease in patients results from severe stenosis or even complete occlusion of 1 or more brain-feeding arteries, often due to progressive atherosclerosis. The slowly increasing restriction of the blood supply through 1 or more feeding arteries is often compensated for by low-resistance anastomoses of the circle of Willis. In accord, patients experiencing occlusive cerebrovascular disease may be without overt pathologic findings, although they often display a mild to moderate decrease in resting cerebral blood flow (CBF). However, neurologic dysfunction may occur in situations in which the regulation of blood supply to the brain is challenged. Clinically, the impairment of CBF regulation is estimated by measuring the cerebrovascular reserve capacity (CVRC) after injection of acetazolamide. If CVRC is small or abolished, the patient may require therapeutic intervention; however, the only therapeutic intervention to improve blood supply for intracranial hemodynamic cerebrovascular disease currently available is extracranial-intracranial bypass surgery.

Under physiologic as well as pathologic conditions, vascular networks undergo a permanent adaptation to chronic changes in the blood supply and/or demand in blood flow. Adaptation may be achieved by 3 major mechanisms: vasculogenesis, angiogenesis, and arteriogenesis. Vasculogenesis is the earliest morphogenetic form of vascular (re)modelling. It is mostly seen in embryonic tissue but has also been shown in adult organisms, where it is associated with the recruitment of endothelial progenitor cells. Angiogenesis describes the branching and sprouting of new capillaries from preexisting vessels. Tissue hypoxia along with vascular endothelial growth factor and angiopoietins are major factors stimulating angiogenesis.

The term arteriogenesis describes the emergence of arteries and arterioles from preexisting microvessels with connection to an established vascular network. The vascular metamorphosis during arteriogenesis comprises active structural remodelling associated with changes in shear stress and upreg-
lation of adhesion molecules. Activated tissue monocytes, ie, macrophages, have been shown to be closely affiliated with growing arterial collaterals. Granulocyte-macrophage colony-stimulating factor (GM-CSF) has been shown to be important for arteriogenesis by enhancing the number and lifespan of monocytes. This effect has successfully been used in patients with coronary artery disease.

An improvement in the blood supply would also be of utmost importance in the hemodynamically compromised cerebral circulation, and monocytes adhering to the vessel wall have been related to changes in cerebral conductance arteries. Our study was designed to examine the influence of long-term treatment with GM-CSF on cerebral hemodynamics (ie, baseline CBF and CVRC) in a rat model of bilateral carotid artery occlusion (BCO). In view of the known angiogenic and arteriogenic effects of GM-CSF, structural changes were investigated, especially with respect to the capillary bed and precapillary resistance vessels.

Materials and Methods

Male Sprague-Dawley rats (290 to 350 g body weight (BW)) were randomly assigned to 1 of the following groups: BCO and daily injection with GM-CSF (Leucomax, Novartis Pharma; 10 μg/kg SC) diluted in 0.3 mL saline (GM-CSF group); BCO and daily subcutaneous injection with 0.3 mL saline (control group); GM-CSF or saline application started with occlusion of the first carotid artery and lasted until the end of the experiments, ie, 5 weeks after the second carotid artery occlusion.

Bilateral CCA Occlusion

Rats were anesthetized with 2% isoflurane in an 80%/20% air/O2 mixture, and body temperature was maintained at 37°C. Animals received a subcutaneous injection of atropine (Fre senator, 5 μg 100/g BW) and buprenorphine (Temgesic, Essex Pharma; 3 μg/100 g BW) to reduce mucus production and postoperative pain. Carotid artery occlusion was performed as described previously. In brief, the common carotid artery (CCA) was exposed through a midline incision and ligated with a silk suture. A recovery period of 1 week was allowed before occlusion of the second CCA. Sham occlusion was performed in a similar manner but with the silk suture removed before the end of surgery.

Measurement of CVRC

Measurement of CVRC was performed before and 1 and 4 weeks after occlusion of the second carotid artery (5 to 8 animals per group). Animals were anesthetized with chloral hydrate (400 mg/kg BW, IP) and fixed in a stereotactic head holder. The scalp was incised; a hole was drilled 2 mm lateral from the bregma on each hemisphere, leaving the internal bone layer intact; and a laser-Doppler flowmetry (LDF) probe (outer diameter 400 μm) connected to an LDF monitor (DRT4, Moor Instruments) was positioned with a micromanipulator. After baseline measurements were taken, acetazolamide (Diamox, Lederle; 20 mg/kg BW, IP) was injected, and the LDF signal was monitored continuously.

Histology and Immunohistochemistry

Five weeks after the second CCA occlusion the aorta was cannulated, and after a blood sample was taken for leukocyte count, the aorta was perfused with a bolus of Evans blue (Sigma-Aldrich; 2% dissolved in saline, 1 mL). After a 90-second perfusion period, the rats were killed and the brains were removed and frozen. From each brain, 20-μm-thick coronal sections were taken 5.2 mm behind the bregma and stained with a hematoxylin/eosin standard procedure. Neuronal damage was investigated in hippocampal regions (CA1, CA3) by evaluating cell density in observation fields (×20 objective) and searching for shrinkage of neurons (×100 objective) with a light microscope (Axioplan 2, Zeiss) equipped with a high-pressure mercury lamp (HBO100, Zeiss) and a digital camera (Axiocam, Zeiss).

Additional sections were taken at the levels of ±0.0 mm, ±2.5 mm, and ±6.0 mm relative to the bregma for the evaluation of capillary density and expression of α-smooth-muscle actin (α-SMA) by immunohistochemistry. For evaluation of capillary density, sections were observed by incident fluorescence microscopy (×20 objective). Areas of interest were recorded. The microvessel density was determined in multiple cortical and subcortical brain areas. Capillaries were selected on the basis of round shape or a transverse line with a maximum diameter of 8 μm.

For α-SMA immunohistochemistry, sections were air-dried and incubated with an alkaline phosphatase–conjugated mouse monoclonal antibody against α-SMA (Sigma-Aldrich, dilution 1:100) for 2 hours. Immune complexes were detected with a fuchsin substrate chromogen system (Dako), and sections were counterstained with hemalum. Each section was scanned (×10 objective), and the numbers of α-SMA–positive vessels were counted on the cortical surface and in the parenchyma.

For CD-68 immunohistochemistry, additional sections were taken from the animals in another set of experiments (3 sections per animal, 4 animals per group) 1 and 5 weeks after the second CCA occlusion. Staining was performed on freshly cut coronal cryosections (thickness 20 μm) postfixed with 4% paraformaldehyde. Sections were pretreated with 0.3% Triton X-100 and 3% H2O2 in 0.3% Triton X-100 for 30 minutes and 60 minutes at room temperature, respectively, followed by blocking with 10% horse normal serum in 0.3% Triton X-100 for 1 hour at room temperature. Incubation was performed with the primary antibody (mouse anti-rat CD-68, Serotec GmbH, Düsseldorf, Germany; dilution 1:100) in 2% rat serum (containing 0.3% Triton X-100) for 24 hours at 4°C followed by incubation with the biotinylated secondary antibody (horse antimouse, Vector, Burlingame, Calif; dilution 1:200) for 1 hour at room temperature. Thereafter, visualization with avidin-biotin complex for 1 hour and horseradish peroxidase substrate (1 minute) was completed. The sections were counterstained with hemalum and scanned for CD-68–positive cells with ×20 and ×40 objectives.

Visualization of Brain Angioarchitecture by Latex Perfusion

In a separate set of animals (4 per group), the brain angioarchitecture was visualized with the latex perfusion technique for cerebral vessels as described previously. The aorta was cannulated with perfluorethenepropylene tubing (internal diameter, 0.76 mm; Vasofix Braunuele, Braun), and a sublethal dose of papaverine hydrochloride (50 mg/kg) was injected to produce maximal vasodilatation. A white latex casting compound (Chicago Latex Products) was mixed with 20 μL/mL carbon black (Derussol N25/L, Degussa) diluted 10:2 with saline and infused into the aorta at an injection pressure of 150 mm Hg. After initiation of infusion, the vena cava was incised to allow venous outflow. Five minutes after the start of perfusion, the animal was transferred to ice-cold water for 20 minutes with injection pressure maintained. Thereafter the brain was removed carefully, and photographs were taken from the base and the top view under an operating microscope with a ×16 objective. Leptomeningeal anastomoses between the anterior cerebral artery (ACA) and the middle cerebral artery (MCA) on both dorsal hemispheres were located and counted by tracking the peripheral branches of the ACA and MCA to the point of confluence. The diameters of the leptomeningeal anastomoses and the diameters of the internal carotid artery (ICA), ACA, MCA, posterior cerebral artery (PCA), posterior communicating artery (PCoA), and basilar artery were measured with freeware image analysis software (Scion Imaging).

Measurement of Baseline CBF by IAP Autoradiography

In a separate set of experiments, rats (4 to 6 per group) were anesthetized with 2% isoflurane 5 weeks after occlusion of the second CCA, and 1
Figure 1. Regional CBF under resting conditions measured by IAP autoradiography at different brain levels revealed no major differences between the experimental groups (A). The right-hand scale indicates CBF in mL/100 g·min⁻¹; the left-hand scale indicates distance from the bregma. For detailed analysis see Figure 1B.
femoral artery and vein were cannulated. Thereafter the animals were placed in a restrainer (Braintree Scientific) and allowed to recover from anesthesia for 1 hour under control of arterial blood pressure and blood gases. Animals received a bolus injection of 1 mL/kg BW Evans blue solution (2% dissolved in saline). One minute later, 125 μCi/kg BW iodo[14C]antipyrine (IAP, Biotrend) was infused at an increasing infusion rate for 1 minute as described elsewhere.15–17 During the infusion, 12 to 16 timed arterial blood samples were taken to determine the time course of the arterial IAP concentration. At the end of infusion, the animals were decapitated and the brains removed and frozen in prechilled isopentane. The brains were embedded in M-1 embedding matrix (Lipshaw), and coronal sections were cut in a cryomicrotome (thickness 20 μm). Sections were exposed together with a [14C] standard set on Kodak MRDM-1 X-ray film for 14 days.

Regional CBF was calculated from the optical densities of the different structures and the time course of the concentration of the radioactivity in the blood by using a brain/blood partition coefficient of 0.9. Optical densities were measured in individual regions of interest with an image analysis system (MCID, Imaging Research Inc).

Data Analysis
All data are given as mean±SD. Changes in LDF were calculated in percent of mean baseline value. With the exception of the number of leptomeningeal anastomoses, statistical analysis was performed by 2-way repeated-measures ANOVA. Pairwise comparison of means was subsequently performed with the Fisher least significant difference test. To compare the numbers of leptomeningeal anastomoses, 1-way repeated-measures ANOVA on ranks with a subsequent Student-Newman–Keulstest for multiple comparisons was performed. Values of \( P<0.05 \) were considered statistically significant.

Results
Physiologic parameters including mean arterial blood pressure (95±8 mm Hg) and blood gas analysis (pH 7.45±0.028, Po2 100±11 mm Hg, Pco2 43±3 mm Hg, and hematocrit 43±2%) did not differ between the individual treatment groups. White blood cell count showed an increase in GM-CSF–treated animals (GM-CSF group 13.7±4.1×10⁹/L, control 6.3±0.2×10⁹/L, and sham 8.3±1.1×10⁹/L; \( P<0.05 \) for GM-CSF vs control).

After BCO, Baseline CBF Is Slightly Decreased, but CVRC Is Abrogated
Five weeks after the second CCA occlusion, mean hemispheric CBF values were 90±8 mL·100 g⁻¹·min⁻¹ in saline-treated animals and 100±9 mL·100 g⁻¹·min⁻¹ in sham-occluded animals (Figure 1). Slightly lower perfusion values were seen in the majority of individual areas reaching statistical significance in the dentate gyrus; however, these alterations did not significantly affect mean hemispheric CBF.

Before BCO, application of acetazolamide increased CBF by 16.4±8.1% without differences between the treatment groups. One week after occlusion of the second CCA, the CBF response to acetazolamide was virtually abolished in control animals (2.0±4.0%, \( P<0.05 \) vs baseline) and did not recover after 5 weeks (2.2±3.7%, \( P<0.05 \) vs baseline). CVRC remained unchanged in sham-operated animals (Figure 2).

We did not find any difference in cell density between the treatment groups in hippocampal CA1 and CA3 regions (CA1 in

Figure 2. Typical LDF response to 20 mg/kg BW acetazolamide in control animals before (A) and after (B) BCO. C. CVRC measured by the LDF response to acetazolamide before and 1 and 4 weeks after the second BCO with a 1-week delay between right- and left-sided carotid artery occlusion. Whereas CVRC of GM-CSF–treated animals (n=5) recovered until the fifth week of treatment, CVRC of control animals (n=5) remained lowered (\# \( P<0.05 \) vs baseline, ## \( P<0.005 \) vs baseline and 5 weeks, ** \( P<0.01 \) vs baseline and 5 weeks, *** \( P<0.01 \) vs control). CVRC remained unchanged in sham-operated animals (n=6).
GM-CSF Attenuates Functional Impairment of CVRC

Long-term treatment with GM-CSF resulted in slightly better perfusion values in 23 of the 27 areas studied by IAP autoradiography, with significantly higher values in the sensory, parietal, and auditory cortices. However, these increases were not high enough to result in a significantly higher mean hemispheric perfusion (102 ± 14 mL·100 g⁻¹·min⁻¹) than in saline-treated animals. One week after the second CCA occlusion, CVRC was virtually abolished in rats treated with GM-CSF as it was in saline-treated animals (4.7 ± 6.1%, P < 0.01 vs baseline). However, after 5 weeks of treatment with GM-CSF, the acetazolamide-induced CBF response had fully recovered (17.2 ± 9.3%, P < 0.01 vs control animals, Figure 2), indicating that GM-CSF treatment restored impaired CVRC.

Effects of Long-Term GM-CSF Treatment on the Microvascular Network

Counting the Evans blue–filled capillaries in different brain areas revealed slightly higher values in mean capillary density in animals treated with GM-CSF (GM-CSF = 280 ± 33 counts/mm², control = 265 ± 22 counts/mm², and sham = 260 ± 14 counts/mm²; P < 0.05 for GM-CSF vs sham); however, the effect was too small to reach statistical significance in individual brain areas (Figure 3). We further determined the density of leptomeningeal and intraparenchymal microvessels immunopositive for α-SMA. Figure 4 shows a characteristic staining pattern of intraparenchymal (Figure 4A) and leptomeningeal (Figure 4B) microvessels. In GM-CSF–treated animals, there was a markedly higher number of intraparenchymal vessels per section (GM-CSF = 191.5 ± 47.1%, saline = 136.3 ± 30.5, and sham = 117.1 ± 23.9, *P < 0.001 vs saline and sham) as well as leptomeningeal vessels per section (GM-CSF = 80.3 ± 7.2%, saline = 63.6 ± 5.1, and sham = 60.1 ± 5.5, *P < 0.01 vs saline and sham). There was no difference between the right and left hemispheres. However, the increase in α-SMA–positive microvessels was found to be more pronounced in the frontal sections (+53% vs control) than in the more occipital sections (29% and 23%, respectively, vs control).

Visualization of the cerebral angioarchitecture by latex perfusion demonstrated an increased diameter of the basal brain vessels in GM-CSF–treated and control animals (see Figure 5A). The mean diameter of the leptomeningeal anastomoses was unchanged (GM-CSF = 35 ± 0.8 μm, control = 33.7 ± 0.7 μm, and sham = 38.9 ± 7 μm). However, there was marked remodelling of the leptomeningeal microvessels...
as demonstrated in Figure 5B (top). This resulted in a significant increase in the number of leptomeningeal anastomoses countable between the ACA and MCA in GM-CSF–treated animals (GM-CSF/H11005 40/H11006 2.6*, control/H11005 31/H11006 2.2, and sham/H11005 28.7/H11006 1.7, *P/H11021 0.05 vs control and sham).

CD-68–Positive Macrophages Are Colocated to the Microvascular Network

Arteriogenesis has been suggested to be mediated by the recruitment of monocytes/macrophages in the perivascular space. We therefore performed CD-68 immunohistochemistry of rat brain specimens 1 and 4 weeks after the second CCA occlusion. One week after the second CCA occlusion, the number of CD-68–positive cells was found to be higher within the perivascular space of GM-CSF–treated animals, whereas fewer CD-68–positive cells were detected in saline-treated controls (Figure 6). Four weeks later, at the end of the experiments, there was still a significantly greater number of cells displaying CD-68 immunoreactivity associated with the vasculature in animals that had undergone BCO. However, there was no marked difference between the animals treated with saline and GM-CSF.

Discussion

In the present study, BCO was performed to mimic the syndrome of occlusive cerebrovascular disease. The main findings of the study are as follows: (1) baseline CBF is not impaired, but CVRC is completely abolished even weeks after BCO, (2) long-term treatment with GM-CSF results in a recovery of CVRC, and (3) this recovery is accompanied by changes in the microvascular network beyond the circle of Willis, most notably on the precapillary arteriolar level.

We used a 2-vessel occlusion model in rats in which both CCAs were permanently occluded at a time interval of 1 week. With this protocol, all animals survived the surgical procedures without any apparent signs of neurological damage. In contrast, if both carotid arteries are occluded simultaneously, brain ischemia along with neurological deficits and even death will develop in a considerable number of animals.13,18 After occlusion of the second CCA, cerebral perfusion is essentially maintained through a single inflow channel, the basilar artery. Five weeks after establishing BCO, resting CBF was decreased in 25 of 27 brain areas as measured by IAP autoradiography. The decrease was mild in
most instances, resulting in a nonsignificant attenuation of mean hemispheric CBF. In accord, there were no signs of neuronal damage in the hippocampal areas CA1 and CA3, the former being the most vulnerable to hypoxic or ischemic damage. These results suggest that the BCO performed in the present study results in a sustained mild impairment of CBF without apparent ischemia and thus, largely reflects the clinical picture of occlusive cerebrovascular disease.

One week after occlusion of the second CCA, CVRC was abolished in all animals. This situation persisted in saline-treated control animals until the end of the experiment, suggesting that adjustment of an almost-normal resting CBF level was accomplished by using up the CVRC. In animals that received long-term treatment with GM-CSF, CVRC recovered and reached the same level as in sham-occluded animals at the end of the observation period. A similar recovery of CVRC after GM-CSF treatment has recently been reported by Buschmann and coworkers in a 3-vessel occlusion model. This recovery was associated with a significant increase in diameter of the PcomA, which is part of the circle of Willis, although arterial enlargement has also been observed in untreated animals. These vascular changes were
considered to reflect ongoing arteriogenesis in the hypoperfused brain. Using the latex perfusion technique, we were able to confirm the results of a significantly increased diameter of basal brain arteries after BCO in saline-treated control animals. In contrast to the study of Buschmann et al.,12 in our model of BCO not the PCA but the PComA displayed the most pronounced increase in diameter after treatment with GM-CSF. However, in light of the profound increase in all basal arteries that failed to improve cerebral hemodynamics in control animals, it seemed unlikely that the additional increase of the PComA completely restored the cerebral hemodynamics in GM-CSF–treated animals. Furthermore, the anterior and posterior circulations are interconnected by both the PComA and the P1 segment of the PCA. The fact that the P1 segment is longer than the PComA but smaller in diameter further underscores the hypothesis that the restored CVRC in the anterior circulation is not due solely to the additional GM-CSF–induced increase in diameter of the PComA. Therefore, we looked for alterations of the cerebrovascular network beyond the circle of Willis. In view of the known angiogenic activity of GM-CSF,20 we determined the capillary density in different brain areas. However, there was only a very mild increase of capillary density in GM-CSF–treated animals. Next, we stained sections for smooth muscle–specific α-SMA to study the precapillary microvessels. Expression of α-SMA has been demonstrated with early angiogenic process microvessel maturation, especially in arterioles.21,22 This seemed to be of particular importance because preexisting collateral arterioles have been previously described as “the substrate of arteriogenesis.”9,23 We found an increased number of vessels displaying α-SMA immunoreactivity comprising both the intraparenchymal as well as the extraparenchymal vessels, the latter located on the cortical surface.

These extraparenchymal, so-called leptomeningeal or pial, arteries are of major importance for regulation of cerebral perfusion, because more than half of the total cerebrovascular resistance is located in the extraparenchymal vessel segments.24 They may form extensive collaterals with each other, thus providing a network of anastomoses between the major brain vascular territories supplied by the ACA, MCA, and PCA. Therefore, an increased number of pial arterioles may indicate the presence of a second network of collaterals distal to the circle of Willis and interconnecting the different hemispheric vascular territories. Evidence in favor of an increased formation of pial collaterals in the hemodynamically compromised rat brain has previously been presented by LDF and subtraction angiography to show the formation of new blood vessels.25 To confirm the described increase in α-SMA–positive arterioles and to gain further insight into the structural changes of the leptomeningeal anastomoses, we scanned the hemispheric surface of latex-perfused brains. Like Buschmann et al.,12 we did not find a significant change in mean vessel diameter of the leptomeningeal anastomoses, but a significantly higher number of those pial vessels could be found after long-term treatment with GM-CSF. A higher number of arterioles will increase the total cross-sectional area and thus, the total conductance of the resistance segment in the organ vasculature. In agreement with this concept is the observed recovery of CVRC obtained by long-term treatment with GM-CSF in the present study.

There is good evidence in favor of GM-CSF’s ability to induce/promote arteriogenesis by amplifying the function of macrophages and monocytes. One week after occlusion of the second CCA, CD-68–positive cells were found closely affiliated with the cerebral microvessels, with a distinct increase in the number of immunoreactive cells in GM-CSF–treated

Figure 6. Immunohistochemical staining for CD-68 1 week after occlusion of the second carotid artery showed vessel-associated immunoreactivity (arrowheads), which was more distinct in GM-CSF–treated animals than in sham or control animals (bar=50 μm).
animals. After 5 weeks of treatment, we still observed a significantly increased number of CD68-positive cells associated with microvascular structures after BCO, however without significant differences between saline- and GM-CSF–treated animals. We therefore propose the early phase after BCO to be crucial for macrophage-induced vessel remodelling. This observation is in accord with previous studies in which an increased number of vessel-associated macrophages was observed after treatment with GM-CSF.10,12

In conclusion, the present study is the first to show that long-term treatment with GM-CSF in a model of cerebrovascular occlusive disease stimulates the formation of extraparenchymal and intraparenchymal arterioles, which will presumably enhance collateralization distally to the circle of Willis and decrease total peripheral resistance. These structural modifications parallel functional improvements, including a recovery of CVRC. Thus, our results imply a significant expansion of the concept of arteriogenesis and its possible therapeutic importance in modulating the response of the cerebral vasculature to chronic hypoperfusion.

Disclosures

None.

References

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