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Progressive expression of vascular endothelial growth factor (VEGF) and angiogenesis after chronic ischemic hypoperfusion in rat

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Summary

Cerebrovascular stenosis caused by arteriosclerosis induces failure of the cerebral circulation. Even if chronic cerebral hypoperfusion does not induce acute neuronal cell death, cerebral hypoperfusion may be a risk factor for neurodegenerative diseases. The purpose of this study was to determine if vasodilation, expression of VEGF, and neovascularization are homeostatic signs of cerebral circulation failure after permanent common carotid artery occlusion (CCAO) in the rat.

Neuronal cell death in neocortex was observed 2 weeks after CCAO and gradually increased in a time-dependent manner. The diameter of capillaries and expression of VEGF also increased progressively after CCAO. Moreover, we observed unusual irregular angiogenic vasculature at 4 weeks.

In conclusion, chronic hypoperfusion results in mechanisms to compensate for insufficiency in blood flow including vasodilation, VEGF expression, and neovascularization in the ischemic region. These results suggest that angiogenesis might be induced in adult brain through the support of growth factors and transplantation of vascular progenitor cells, and that neovascularization might be a therapeutic strategy for children and adults with diseases such as vascular dementia.

Keywords: Global ischemia; hypoperfusion; vascular endothelial growth factor; angiogenesis.

Introduction

Cerebrovascular stenosis caused by arteriosclerosis induces cerebral circulation failure. Even if chronic cerebral hypoperfusion might not induce acute neuronal cell death, magnetic resonance imaging and positron emission tomography suggest it participates in the development of vascular dementia [4, 10, 12, 20, 24, 31]. Moreover, it has been reported that cerebral hypoperfusion occurs in Alzheimer's disease and Binswanger's disease (subcortical arteriosclerotic encephalopathy) [2, 8, 15, 26, 30]. Therefore, cerebral hypoperfusion is suggested as a risk factor for neurodegenerative diseases.

Therapeutic angiogenesis is a strategy where blood vessel formation is induced for the purposes of treating and/or preventing ischemic disease such as myocardial, hind-limb, and cerebral ischemia [7, 9, 22, 23, 32]. Moyamoya disease, a cerebrovascular disease that occurs mostly in children, features angiogenesis in the brain and an increase of growth factors in the cerebrospinal fluid [19, 21, 28, 29, 34]. Angiogenesis (neovascularization) is thought to be induced by chronic cerebral hypoperfusion and, thus, is a homeostatic sign of failure of the cerebral circulation that is involved in compensation for the impaired circulation. Neovascularization through surgery is one therapeutic strategy to compensate for impaired circulation in Moyamoya disease [6, 7, 17].

It is still uncertain whether therapeutic neovascularization is appropriate for adult cerebral hypoperfusion. The purpose of this study was, therefore, to measure vasodilation by vessel diameter and the expression of vascular endothelial growth factor (VEGF) as homeostatic signs of cerebral circulation failure in young adult rats during chronic hypoperfusion.

Materials and methods

Production of permanent forebrain ischemia

Male Slc/Wistar rats aged 13 to 15 weeks (SLC, Shizuoka, Japan) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Anesthetized rats were laid on their back and a midline neck incision made. The common carotid arteries (CCAs) were carefully exposed and isolated. Then, the CCAs were doubly ligated with 3-0 silk suture and the arteries cut between the sutures (CCA occlusion; CCAO). After the surgical operation, the rats were maintained under

an infrared heat lamp until awake to avoid a decline in body temperature. All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of Showa University.

Histology and measurement of capillary diameter

At 0, 1, 2, and 4 weeks after ischemia, the animals were re-anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and the brains were removed. The brains were immediately immersed in 10% buffered formalin for fixation for 1 week and decalcified with 1% formic acid. After embedding in paraffin, 10 μm sections were cut. These were deparaffinized and used for either hematoxylin and eosin staining for morphological evaluation, or for VEGF and factor VIIIa (FVIIIa, a vascular marker) immunostaining. After boiling in 10 mmol sodium citrate buffer (pH 6.0) for 15 minutes, the sections were preincubated in 0.3% H_2O_2 to inhibit endogenous peroxidase activity. After washing, the sections were incubated in normal goat serum to block the non-specific reaction and were then incubated with a polyclonal rabbit anti-VEGF (Ab-2) antibody (1 : 100; Oncogene Research Products, Cambridge, MA) or rabbit anti-human FVIIIa polyclonal antibody (1 : 200; DakoCytomation, Glostrup, Denmark). They were developed with Histofine SAB-PO (R) kit (Nichirei, Tokyo, Japan) with diaminobenzidine (DAB) as the chromogen. Sections were counterstained with hematoxylin after the DAB reaction for cell identification. After staining, the sections were examined and images taken with the aid of light microscopy (Olympus AX-70; Olympus, Tokyo, Japan).

Capillary diameters in hippocampus and neocortex were measured in a coronal section from the bregma (−1.0 to −5.0 mm) immunostained for FVIIIa. Images (440 × 320 μm^2) were examined and the diameters of 5 to 7 capillaries within that area were measured. A total of 64 to 81 (n = 3–4 animals) capillaries were measured in each region of the hippocampus and neocortex at 0, 1, 2, and 4 weeks after CCAO. The quantitative determination of capillary diameter was performed using NIH Image, version 1.62 (National Institutes of Health, Bethesda, MD).

All data are expressed the mean ± standard error. Statistical comparisons were made using Dunnet's post hoc test followed by one-way ANOVA as compared to sham-operated control (0 week). A p-value of < 0.05 was considered statistically significant.

Results

Progressively increased neuronal cell death in cerebral cortex (Fig. 1)

In the absence of hypotension, occlusion of CCAs generally does not cause neuronal cell death. However, it is reported that CCAO in Slc/Wistar rats induces neuronal cell death due to the patency of the posterior communicating arteries [16]. One week after CCAO, approximately 30% of animals died and 36.4% (4 of 12) of the surviving animals showed neuronal cell death in the hippocampal CA1 region. The percentage of animals with neuronal cell death in the hippocampus increased to 57.1% (4 of 7) at 2 weeks and did not increase further at 4 weeks. In contrast, there was no marked neuronal cell death observed in the neocortex

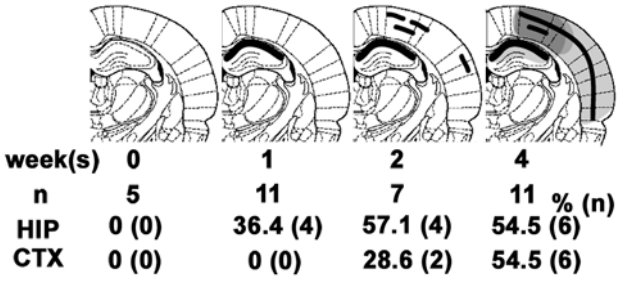

Fig. 1. Morphological changes in hippocampus (*HIP*) and neocortex (*CTX*) after chronic cerebral hypoperfusion induced by 0, 1, 2, or 6 weeks of permanent CCAO. There was a time-dependent gradual increase in neuronal cell damage in HIP and CTX, as indicated by *gray* and/or *black*. The number of animals examined (*n*) and percentage of animals with HIP and CTX neuronal damage (absolute number in parentheses) is given. By 4 weeks after CCAO, 50% or more of surviving animals had neuronal cell death in HIP and CTX

1 week after CCAO. Neuronal cell death in the neocortex gradually increased to 28.6% (2 of 7) and 54.5% (6 of 11) at 2 and 4 weeks after CCAO, respectively.

Homeostatic signs of failure of cerebral circulation

Vasodilation is thought to compensate for insufficient blood flow in brain [13]. Thus, we determined the diameter of capillaries in the hippocampus and neocortex following CCAO (Fig. 2A). The average capillary diameters were 1.7 ± 0.07 and 1.6 ± 0.05 μm in sham-operated controls (0 week) in hippocampal and neocortical regions, respectively. After ischemia, capillary diameter clearly increased and the diameter was significantly greater than pre-ischemia values 1 week after CCAO. Four weeks after CCAO, the diameters of capillaries in the hippocampus and neocortex were 2.4 (4.1 ± 0.29 μm) and 2.5 (4.0 ± 0.19 μm) fold greater than sham-operated controls, respectively.

VEGF expression was examined using immunohistochemistry. Few immunopositive reactions for VEGF were observed preischemia (Fig. 2B). After ischemia, the positive reaction for VEGF was increased in a time-dependent manner and was obvious adjacent to the vasculature (Fig. 2C–E). Immunopositive cells for VEGF were mainly observed around the infarction, but not within the infarction. VEGF was expressed in astrocytes, as demonstrated by double-immunostaining (data not shown).

VEGF is well known to participate in angiogenesis [5, 22]. Therefore, the angiogenic vasculature was determined by staining for FVIIIa. As shown in Fig. 2F and G, an unusual irregularly-constructed vasculature

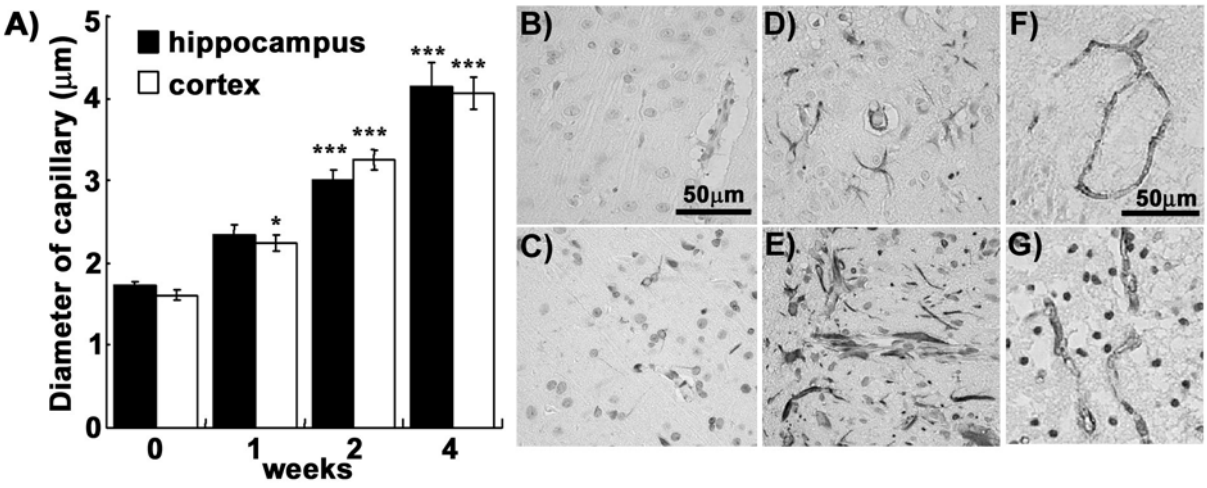

Fig. 2. Homeostatic sign of failure of cerebral circulation after chronic cerebral hypoperfusion. (A) Semi-quantification of mean capillary diameter in hippocampal (*black bar*) and neocortical (*white bar*) regions. There was a time-dependent increase in capillary diameter in both regions. Data are presented as mean ± SE. * = $p < 0.05$, *** = $p < 0.001$, as compared with sham operated control (0 week) using by Dunnett's post hoc test followed by one-way ANOVA. (B–E) VEGF-like immunoreactivity (ir) 0 (B), 1 (C), 2 (D), and 4 (E) weeks after CCAO. VEGF-ir progressively increased in a time-dependent manner. VEGF-ir was observed adjacent to the capillary as shown in (E). (F) and (G) show the brain microvasculature 4 weeks after CCAO. Unusual irregular constructed vasculature such as loops (F) and meandering (G) were often noted at 4 weeks after CCAO. This suggests that neovascularization was occurring in the brain parenchyma during chronic hypoperfusion

(e.g., loops and meandering vessels) was often noted in the peri-infarct region of cerebral cortex 4 weeks after CCAO. These were angiogenic vasculature. This implies that neovascularization was increased in the brain parenchyma during forebrain ischemia.

Discussion

The present study indicates that there is sustained vasodilation and expression of VEGF after chronic forebrain ischemia in our rat model. Moreover, we also found evidence of neovascularization by FVIIIa immunostaining at 4 weeks after CCAO.

Numerous reports on rodents, such as gerbil, rat, and mouse, have shown that global ischemia induces neuronal cell death in the hippocampus 2 or 3 days after induction [11, 14, 18, 33]. Such cell death is known as delayed neuronal cell death and is considered a target for therapy of cerebrovascular diseases. However, in most strains of rats, CCAO alone does not induce neuronal cell death due to the patency of the posterior communicating artery and few papers have reported cortical neuronal cell death in rat global ischemia in the chronic phase [1, 3, 16, 33]. Slc/Wistar rat is one strain of rats where cortical neuronal cell death is induced by CCAO alone [16]. In the present study, we reconfirmed that CCAO induces neuronal cell death in the hippocampus and neocortex in that strain. In particular, neocortical neuronal cell death was observed 2 weeks after CCAO and the infarction gradually extended in a time-dependent manner. At 4 weeks after CCAO, the neocortex had evident parenchymal atrophy and proliferation of fibroblast-like cells in the core of the infarction (data not shown). These results indicate that the CCAO model in Slc/Wistar rats might be a good model for studies of chronic hypoperfusion, such as vascular dementia.

We found that both the diameter of capillaries and the expression of VEGF increased in the ischemic region during the 4 weeks after CCAO. We hypothesize that this is a compensatory response to insufficiency of blood flow in brain. In addition, at 4 weeks after CCAO, there was evidence of revascularization in the ischemic region.

Injection of VEGF induces angiogenesis after cardiac, hind-limb, or cerebral ischemia [9, 23, 27, 32]. On the other hand, VEGF has also been reported to increase vascular permeability and inflammatory responses [5, 22, 25]. Although we need to clarify the relationship between VEGF and angiogenesis further, we hypothesize that neovascularization occurs in the adult brain to increase blood flow to ischemic regions during chronic hypoperfusion.

In conclusion, this study provides evidence that chronic brain hypoperfusion elicits mechanisms to compensate for the insufficiency of blood flow, i.e., it induces vasodilation, VEGF expression, and neovascularization in the ischemic region. These results suggest that angiogenesis might be induced in the adult brain by the application of growth factors and transplantation of vascular progenitor cells. Thus, neovascularization might be a therapeutic strategy for children and adults with diseases such as vascular dementia.

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