Sequential and spatial profiles of apoptosis in ischemic penumbra after two-vein occlusion in rats

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Object. The two-vein occlusion model is known to be useful for ischemic penumbra studies in vivo. It was applied here to examine sequential changes in the expression of Bax and Bcl-2 proteins and in apoptotic cells to assess the relationship between penumbra and apoptosis.

Methods. Two cortical veins were occluded photochemically by using rose bengal dye in 27 Wistar rats. The animals were killed with perfusion fixation at the following intervals: 4, 12, 24, 48, 96, and 168 hours after vein occlusion (four at each interval; three additional rats were sham-treated). Immunohistochemical analysis for the Bcl-2 family of proteins was performed along with the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay to examine the relationship to single-cell death.

Cells positive for antia apoptotic proteins began to appear in the TUNEL assay for animals killed 24 hours after vein occlusion, with a peak at 48 hours. These cells were localized in the core of infarction. Immunohistochemical staining for Bax protein showed an increased presence around ischemic lesions at 4 hours after vein occlusion, and the amounts continued to rise until 24 hours, when the localization was diffuse around the core of infarction. Negative findings on immunohistochemical studies for Bcl-2 protein were seen at the early phase after two-vein occlusion.

Conclusions. After vein occlusion, apoptosis appeared sequentially and widely in cortical lesions considered to be the penumbra. Therefore, control of apoptosis would be expected to offer a therapeutic window for treatment of venous infarction.

Key Words • two-vein occlusion • apoptosis • terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling • antia apoptotic protein • rat

VENOUS infarcts are uncommon and frequently misdiagnosed as arterial infarcts, intracerebral hemorrhage, or tumors. Currently, there is considerable interest in brain injury caused by cerebral venous circulation disorders. This is a result of the increasing number of neurosurgical procedures for elderly patients and the development of skull base neurosurgery. Nevertheless, the pathophysiological features and treatment of cerebral venous infarction remain uncertain both clinically and experimentally. Due to the inconsistent symptoms and rarity of venous infarction, this condition has been frequently misdiagnosed.

The term “ischemic penumbra” has been used to define regions in which CBF reduction passes the threshold that leads to the failure of electrical but not membrane function. The penumbra is thus a potentially treatable zone and a spatially dynamic brain region with limited viability, which is characterized by complex pathophysiological changes. A large amount of evidence suggests that ischemic penumbra occurs in animals and humans after focal brain ischemia. In experimental animals, the pathophysiological features of the penumbra have been studied predominantly in rat models with occlusion of the MCA. Recently, photochemical occlusion of two cortical veins in rats was proposed as an animal model of penumbra with distinct advantages. This model leads to a rather widespread reduction in cortical flow and the development of small infarcts. Therefore, it allows for studies of cerebral low-flow conditions similar to those expected to occur in an ischemic penumbra zone.

Apoptosis, or programmed cell death, plays an important role in many developmental and pathological processes of the central nervous system, and it is believed that cells in the penumbra die of both necrosis and apoptosis. Therefore, survival of cells in the penumbra may be promoted not only by reperfusion but also by interrupting the process of commitment to apoptosis. This approach holds promise as a future therapeutic strategy for venous infarcts.

The Bcl-2 factor is one that performs an important role during the process of apoptosis. Functionally, Bcl-2 is recognized as an effective factor for restraining apoptosis. Identification of Bcl-2 proteins and several genes that constitute the Bcl-2 family has been reported. Many of the proteins in this family, similarly to Bcl-2, inhibit apoptosis to prevent cell death. Nevertheless, other proteins in the Bcl-2 family, such as Bax, promote apoptosis and induce cell death. Thus, the temporal appearance of Bcl-2 family pro-
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teins indicates a trigger in the form of either promotion or inhibition of apoptosis. Furthermore, it has been reported that ischemic neuron death is related to the functioning of the Bcl-2 family.6,9

The experiment reported here was designed to elucidate pathophysiological events occurring during apoptosis in venous ischemia. We examined sequential changes in the Bcl-2 family of proteins and in the apoptotic cells to assess the correlation between penumbra and apoptosis in the rat two-vein occlusion model.

Materials and Methods

Animal Preparation

Twenty-seven male Wistar rats (each weighing 280–380 g) were anesthetized by intraperitoneal injection of chloral hydrate (36 mg/100 g body weight) after premedication with 1 mg atropine. Anesthesia was maintained using chloral hydrate (12 mg/100 g body weight) administered hourly through a peritoneal catheter under conditions of spontaneous breathing. Rectal temperature was maintained at 37°C with a feedback heating pad (CMA 150; Carnegie Medicine AB, Stockholm, Sweden). Polyethylene catheters were inserted into the tail artery and left femoral vein. The arterial line was used for continuous monitoring of mean arterial blood pressure and arterial blood gas sampling, and the venous line was used to administer fluid and drugs. The PaO₂, PaCO₂, and arterial pH were measured (ABL 330; Radiometer, Copenhagen, Denmark), and blood pressure was continuously monitored through an intrarterial catheter connected to a pressure transducer.

Rats were secured in stereotactic frames (SR-6; Narishige, Inc., Tokyo, Japan) for the duration of the experiment. After performing a 1.5-cm midline skin incision, a left frontoparietal cranial window was prepared using a high-speed drill for access to the brain surface. The entire procedure was performed with the aid of an operating microscope (Carl Zeiss, Oberkochen, Germany). During the craniectomy, the drill tip was cooled continuously by using physiological saline to avoid thermal injury to the cortex. The dura mater was left intact.

Three sham operations were performed without inducing photothermal thrombosis, and it was confirmed that the intact brain showed no remarkable changes when compared with the nonoccluded side in the animals with thrombosis.

Cortical Vein Occlusion by Photochemical Thrombosis

The occlusion of two adjacent cortical veins was accomplished using rose bengal dye (Katayama Chemicals, Osaka City, Japan) and fiberoptic illumination (L4887 fiberoptic system [6500–7500 lux, 540 nm]; Hamamatsu Photonics, Hamamatsu, Japan) with a 100-μm fiber. Rose bengal dye (50 mg/kg) was injected slowly without affecting the systemic arterial pressure, and care was taken to avoid the illumination of tissue and vessels near the target vein, which was exposed for 10 minutes with the aid of a micromanipulator-assisted light guide. To occlude the second selected vein, another 25-mg/kg dose of rose bengal dye was injected intravenously before the illumination was repeated with the new target. Fluorescence angiography was subsequently performed to study the microcirculation of the brain and confirm vein occlusion. Epicortical vessel structures were examined after intravenous injection of 2% Na⁺-fluorescein solution (Nacalai Tesque, Inc., Kyoto, Japan) with an excitation source at a wavelength of 450 to 460 nm (12-filter block; Leitz, Wetzlar, Germany). After confirmation of vein occlusion, the rats were returned to individual cages and allowed free access to food and water.

Histopathological Preparation

At 4, 12, 24, 48, 96, and 168 hours after vein occlusion, groups of four rats were killed with perfusion fixation, which was initiated with a transcardiac infusion of saline, followed by 10% formaldehyde. The brains were excised and coronal blocks from the middle frontal cortex with the two dorsal cerebral veins were processed for embedding in paraffin. Sections measuring 5 μm were cut and stained with H & E. The size and location of infarct lesions were examined.

In Situ Labeling of Apoptotic Cells

The DNA fragmentation of cells undergoing apoptosis was analyzed using the TUNEL method; sections were processed in accordance with the instructions in the ApopTag kit (Oncor, Inc., Gaithersburg, MD) with minor modifications. In brief, digoxigenin-deoxyuridine 5-phosphate was used to label 3’OH ends by terminal deoxynucleotidyltransferase, and digoxigenin was detected by anti-digoxigenin peroxidase labeling and then by the 3',3' diaminobenzidine-HCl reaction. We used the thymus of the rat as a positive control.

The TUNEL-positive, darkly stained nuclei or nuclear fragments with a cytoplasmic halo were recognized as apoptotic-positive cells. During evaluation of the number of apoptotic cells, the presence of standard morphological characteristics of apoptosis in TUNEL-positive cells was confirmed.

Immunohistochemistry for Bcl-2 Family Proteins

For immunohistochemical analysis, sections were deparaffinized and boiled in citrate buffer for 5 minutes at 120°C in an autoclave. Endogenous peroxidase activity was blocked by incubation in 0.3% H₂O₂, and sections were incubated overnight at 4°C with mouse monoclonal antibodies to Bax (SC7480; Santa Cruz Biochemicals, Santa Cruz, CA) and Bcl-2 (SC7480; Santa Cruz Biochemicals); each sample was diluted 1:500. The sections were visualized using an SAB-PO(M) kit (Vector Laboratories, Burlingame, CA) and diaminobenzidine. Finally, counterstaining was performed using hematoxylin.

Histopathological Evaluation

Initially, the lesions that appeared after vein occlusion were evaluated using H & E staining (Fig. 1). Areas in which morphological changes in neurons were detected were considered to be infarction cores, and the surrounding border zone was identified as the penumbra area. Apoptotic death was assessed using the TUNEL method with histological criteria, that is, nuclear pyknosis, chromatin condensation or fragmentation, and cytoplasmic shrinkage. To evaluate TUNEL semiquantitatively, Bax and Bcl-2 staining intensities were scored on the following four-point scale: −, no staining; +, weakly positive; ++, moderately positive; and ++++, strongly positive. Staining intensity was decided after comparison with the contralateral intact side as the control area.

![Fig. 1. Coronal section of a rat brain showing a small infarction 24 hours after two-vein occlusion. The square is located in the infarction core, the circle designates the penumbra area, and the triangle marks the area distant from the infarction center. H & E, original magnification × 40.](image-url)
Results

Physiological Variables

There were no statistical differences in blood gas levels (PaO₂, PaCO₂, and pH) before and after the cortical vein occlusion. The mean arterial blood pressure did not change significantly and remained between 80 and 100 mm Hg.

Histopathological Findings at Each Time Point

As summarized in Table 1, the following histopathological findings were obtained in the six occlusion groups.

The 4-Hour Group. At 4 hours after vein occlusion, TUNEL signals were rare, but a few positive cells were evident in the infarction core. Several neurons already showed moderate cytoplasmic Bax immunolabeling in the infarction core and penumbra area; however, the areas distant from the core were negative. A few Bcl-2 protein molecules were expressed in the infarction core. Immunolabeling of Bcl-2 was not detected in other areas.

The 12-Hour Group. At 12 hours, TUNEL-positive nuclei were detected in the infarction core and to a lesser extent in the penumbra area, but not in other areas. The Bax protein was expressed strongly in the infarction core and moderately in the penumbra area. Furthermore, a few Bax protein molecules were detected around this area. Immunolabeling for Bax showed the tendency to diffuse in a radial pattern at the center of the infarction core (Fig. 2d–f). Results of immunolabeling for Bcl-2 were negative in almost all areas. A few Bcl-2 protein molecules, which were suspected to be a mixture of false-positive cells, were expressed in the infarction core (Fig. 2g–i).

The 24-Hour Group. At 24 hours, numerous TUNEL-positive cells were observed in the infarction core, and moderate numbers were observed around the penumbra area. Moreover, a few positive cells were detected in areas distant from the core. The TUNEL signals also showed a tendency to diffuse in a radial pattern (Fig. 2a–c). These alternations followed the expression of Bax proteins. The Bax protein was expressed strongly in the ischemic center, and in distant areas, weak Bax immunolabeling was apparent. In general, results of immunolabeling for Bcl-2 were still negative.

The 48-Hour Group. At 48 hours, the peak of TUNEL-positive cells was evident in the infarction core and the penumbra area; positive cells were also observed dotting the areas distant from the core. The Bax protein was still expressed strongly in the penumbra and in a distant area as a peak; however, the expression showed a tendency to decrease in the infarction core. Expression of Bcl-2 was unchanged.

The 96-Hour Group. At 96 hours, TUNEL-positive cells were studded far from the core, and a decreasing tendency was observed in the infarction core and penumbra area. The presence of Bax protein was decreased around the ischemic center and was rare in distant areas; however, Bcl-2 protein was apparent around the ischemic core.

The 168-Hour Group. At 168 hours, TUNEL signals were rare, and Bax protein was expressed only weakly. The Bcl-2 protein was expressed moderately around the ischemic core.

Discussion

Venous Ischemia

In our study, a clear sequence of events was revealed after two-vein occlusion in the rat brain. The Bax expression prior to active apoptosis first occurred in the infarction core, followed by the penumbra, and only to a very limited extent in distant areas. This is in keeping with the clinical features of cerebral venous occlusion, which are perturbation of the circulation resulting in local critical ischemia and severe brain damage. In pathophysiological studies, venous occlusion leads to an increase in cerebral blood volume and cerebral fluid content, resulting in intracranial hypertension and decreased regional CBF as well as long-term changes in brain function. Otsuka, et al. demonstrated that the greater vulnerability to cerebral venous circulation disorders in the aged brain might be attributed to early and extensive hypoperfusion of the circumscribed brain areas drained by the occluded vein. Nagata, et al. demonstrated that, compared with vein occlusion or brain compression alone, the accumulated episode caused severe ischemia, then increased the vulnerability of the rat brain to tissue damage.

Some factors related to cerebral venous circulation disorders and subsequent brain damage were shown in experimental studies. At the time of those studies, however, the

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* - no staining; ±, weakly positive; +, moderately positive; ++, strongly positive.
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Fig. 2. Photomicrographs of cells in sections of ischemic rat brain prepared with TUNEL staining (a–c) and immunolabeling with Bax (d–f) and Bcl-2 (g–i). a: Section showing TUNEL staining in the infarction core 24 hours after two-vein occlusion. Several positively staining cells are observed in the ischemic center. b: In the penumbra area, a few TUNEL-positive cells are present 24 hours after two-vein occlusion. c: At a distance from infarction core, TUNEL-positive cells are rare 24 hours after two-vein occlusion. d: Strong Bax immunostaining is present 12 hours after two-vein occlusion in the infarction core. e: In the penumbra area, Bax immunostaining is moderate 12 hours after two-vein occlusion. f: At a distance from the core, a few positive cells express Bax immunostaining 12 hours after two-vein occlusion. g: Results of immunostaining with Bcl-2 are unremarkable except for false-positive cells 12 hours after occlusion of the two veins in the infarction core area. h: The penumbra area lacks Bcl-2 immunostaining 12 hours after two-vein occlusion. i: At a distance from the infarction core, no Bcl-2-positive cells are evident at 12 hours. Original magnification × 400.

pathophysiological mechanism of neuronal death as the terminal stage of cerebral venous circulation disorders was not known. Our study showed that neuronal death after vein occlusion was related to apoptosis, and the factors that result in vulnerability to cerebral venous circulation disorders may be the signal of apoptosis.

Ischemic Penumbra Zone in Venous Circulation Disorders

The term “ischemic penumbra” has been used to define a region in which CBF reduction has passed the threshold that leads to the failure of electrical but not membrane function.1 With declining flow rates in the brain, protein synthesis is initially inhibited (at a threshold of 0.55 ml/100 g/min), followed by the release of neurotransmitters and disturbance of energy metabolism (at 0.2 ml/100 g/min), and finally the anoxic depolarization that limits the survival of the penumbra (< 0.15 ml/100 g/min).8 The concept underlying this is that in ischemic penumbra, the neurons remain structurally intact; however, it is apparent that the pathophysiological mechanism is affected. The functionally disturbed neurons, however, must be a result of inhibited protein synthesis and release of neurotransmitters, and the transient neurons that survive and the structurally intact ones may change and undergo cell death in the future. This phenomenon may be related to the fact that the penumbra is unstable in both time and space.

There is a large amount of evidence that an ischemic penumbra easily develops in experimental animals and humans after the occurrence of focal brain ischemia.8 In animal experiments, the pathophysiological features of the penumbra are investigated primarily by using rat MCA occlusion models. In the penumbra area, the collateral blood flow or reperfusion would greatly contribute to protection from cell death. If hypoperfusion in the penumbra progresses or is maintained for longer periods, an unstable area becomes irreversibly damaged and eventually leads to cell death. In venous thrombosis, the area of infarction remains small in comparison with the extent of functional disturbance in the penumbra zone.3 The affected area with venous obstruction is complicated by several collateral pathways and the occur-
ence of venous hypertension. In the current experiment, we used photochemically induced two-vein occlusion in rats to observe a widespread area considered to be the penumbra, and confirmed that the slow and extensive change was due to apoptosis.

**Apoptosis and Penumbra**

The general concept of the mechanism of ischemic neuronal death is that it is characterized by cell swelling. In the infarction core, inhibition of adenosine triphosphate synthesis occurs with the formation of nitric oxide radicals that lead to necrosis. Recently, it has been suggested that ischemic signals operate via several cascades in bringing about apoptosis. As shown in this study, progression of an infarct occurs in concert with apoptosis, particularly in the peri-infarct core penumbra. Observations made using the MCA occlusion model demonstrated that in the area of the pen-
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umbra, alteration in Bax protein occurred 3 hours after ischemia. In our study, immunohistochemical staining of Bax/Bcl-2 proteins, which cause apoptosis, appeared at an early stage, that is, 4 hours after vein occlusion. This is in keeping with the observation that Bcl-2 expression did not appear until 7 days after vein occlusion. These phenomena occurred due to the promotion or inhibition by ischemic signals. Maximum Bax immunostaining was observed 24 hours after vein occlusion.

Apoptosis in Arterial and Venous Occlusion

The presence and anatomical location of cells exhibiting DNA fragmentation after MCA occlusion suggests that apoptosis contributes to the development of ischemic infarction. Groups of cells exhibiting DNA fragmentation were located primarily in the inner boundary zone of the infarct. Induction of an apoptosis-related gene after focal ischemia may contribute to cell death in the core and selective cell death adjacent to the infarct. Studies of arterial infarction in which the MCA occlusion model was used showed several characteristics of neuronal cell death. The infarction caused by arterial obstruction shows a clear border zone, and apoptosis is limited to the area around the infarction core (Fig. 3 left). On the other hand, the appearance and changeability of the venous ischemic brain has a very specific variability that is different from arterial ischemia. Venous ischemia is characterized by the course of ischemic change and the wide ischemic penumbra area.

It has previously been reported that venous infarction aggravates circulatory failure and deteriorates further with venous thrombosis. Furthermore, it is reported that a wide reduced-flow area appears after cortical vein obstruction and that cortical spreading depression specific to ischemic penumbra is observed. In addition, we have confirmed that apoptosis due to venous ischemia appeared in the center of the infarct and in remote areas. Congestion of the cerebral vein impairs the microcirculation in the cortical area, and the gradually expanding ischemic area further complicates CBF and unstable brain tissue. The area around the infarction core is an incomplete ischemic stage, and the function and condition of neurons may be observed as a specific change related to the ischemic stage. Venous ischemia, in particular, shows widespread change and gradual alterations after venous occlusion, which is the reason for the widespread appearance of apoptosis after venous ischemia (Fig. 3 right).

Antiapoptosis Treatment in Venous Infarction

In this paper we provides evidence of the occurrence of a sequence of events after ischemic insult due to venous obstructions, with waves of Bax expression and apoptosis initially occurring in the infarction core and then in the penumbra. The latter can be considered as a potentially treatable zone and a spatially dynamic region of limited viability, which is characterized by complex pathophysiological changes but might be amenable to therapeutic intervention.

Conclusions

Venous ischemia induced a rather widespread reduction of cortical flow and resulted in specific neuronal death. After vein occlusion, apoptosis appeared sequentially and widely in cortical lesions considered to constitute penumbra. Venous infarction shows widespread change and gradual alterations in comparison with arterial infarction. Therefore, control of apoptosis would be expected to offer a therapeutic window for treatment of venous infarction.

Acknowledgment

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