Vascular Endothelial Growth Factor Antagonist Reduces Brain Edema Formation and Venous Infarction

R. Kimura, MD; H. Nakase, MD; R. Tamaki, MD; T. Sakaki, MD

**Background and Purpose**—Cerebral venous ischemia often induces severe brain edema. Vascular endothelial growth factor (VEGF), which induces angiogenesis, is also known as vascular permeability (VP) factor. The present study was undertaken to investigate whether the inhibition of VEGF could reduce brain edema formation and cerebral venous infarction (CVI) in a rat 2-vein occlusion (2-VO) model.

**Methods**—We used 2-VO model in which 2 adjacent cortical veins were photochemically occluded. Male Wistar rats (n=25) were divided into 2 groups: one group was treated with a VEGF antagonist (antagonist group, n=10) and the second group was treated with phosphate-buffered solution (PBS) (PBS group, n=15). VEGF antagonist or PBS was injected intraperitoneally immediately after 2-VO. The developing ischemic infarct was evaluated by magnetic resonance imaging (MRI) and histology 24 hours after occlusion.

**Results**—VEGF expression was observed in the cytoplasm of neurons exclusively in the area of vasogenic edema that was induced by hypoxia.8 The VEGF induction is most prominent in the patient’s condition.8,9 VEGF has been suggested as a new therapy for both brain edema formation and CVI. (Stroke. 2005;36:1259-1263.)

**Conclusions**—Our study is the first to provide evidence that the inhibition of VEGF attenuates VP and reduces CVI in the acute stage of cerebral venous ischemia; MRI, immunohistochemistry, and histology were used for the investigation.

**Key Words:** antagonist ■ brain edema ■ cerebral venous ischemia ■ rat ■ vascular endothelial growth factor

**Brain edema** is one of the main complications associated with brain diseases. It is induced by various conditions such as cerebral ischemia,1 physical damage,2 central nervous system infections,3 and brain tumors.4 Brain edema sometimes leads to high intracranial pressure and deterioration of the patient’s condition.

Recently, we revealed vascular endothelial growth factor (VEGF) expression in the brain edema area after cerebral vein occlusion in the rat.5 We evaluated brain edema formation by magnetic resonance imaging (MRI).6 VEGF is an angiogenic factor that was first purified from the ascitic fluid of guinea pigs implanted with hepatocarcinoma cells7 and induced by hypoxia.8 The VEGF induction is most prominent in the cytoplasm of neurons in the ischemic border zone within 24 hours after stroke.9,10 VEGF has been suggested as a new therapeutic mediator for ischemic diseases because of the angiogenetic and neuroprotective effects. However, VEGF also increases vascular permeability (VP), which could increase brain edema. Therefore, in the acute stage of brain ischemia, the effect of VEGF is considered controversial. This study was performed to investigate whether an inhibition of VEGF could reduce brain edema and cerebral venous infarction (CVI) in the acute stage of cerebral venous ischemia; MRI, immunohistochemistry, and histology were used for the investigation.

**Materials and Methods**

This animal study was conducted in accordance with the guidelines approved at the 80th general assembly of the Japan Science Council (1980).

**Animal Preparation**
We used 25 male Wistar rats weighing 230 to 300 grams (CLEA Japan, Inc; Osaka). After premedication with 0.5 mg atropine sulfate, chloral hydrate was injected intraperitoneally (36 mg/100 g weight) for the induction of anesthesia. The anesthesia was maintained using 0.5% to 1.0% halothane in 50% N₂O and 50% O₂ during the surgery. Spontaneous ventilation was maintained during the procedures. The rectal temperature was maintained at ~37.0°C in all animals. A polyethylene catheter was placed into the tail artery and was used for continuous monitoring of the mean arterial blood pressure using a pressure transducer (Polygraph system RM-600; Nihon Koden) and for measuring blood gases (PaO₂, PaCO₂, pH) a blood gas analyzer was used (ABL 330; Radiometer). Each rat was mounted on a stereotactic frame (SR-6; Narishige Inc) and a 1.5-cm midline skin incision and a cranial window (4.5 mm×6 mm) were made over the left parietal region using a high-speed drill. This procedure was performed under an operating microscope (Zeiss). A continuous flow...
of physiological saline cooled the drill tip during this procedure. The dura was left intact, and the left parietal cortex was exposed.

Cortical Vein Occlusion by the Photochemical Thrombotic Technique
Two adjacent cortical veins were photochemically occluded using rose bengal dye and fiberoptic illumination as described previously.5,6,11,12,13

Experimental Design and Groups
Twenty-five rats were randomly divided into 2 groups in blind fashion. The antagonist group was treated with a VEGF antagonist (neutralizing antibody against VEGF in phosphate-buffered solution (PBS), 2 mg/Kg, rabbit polyclonal antibody, RB-222, NeoMarkers); (n=10). The PBS group was treated with PBS (n=15). All the rats received an intraperitoneal injection of either a VEGF antagonist or PBS immediately after the vein occlusion. Both groups were evaluated using MRI and histology 24 hours after surgery.

MRI
The extent of ischemic damage was evaluated in rats by using MRI 24 hours after surgery. The time point was chosen based on earlier data that was obtained from our laboratory.13 Animals were re-anesthetized (2.0% halothane in 100% O2) and placed in a supine position with their heads inside birdcage radiofrequency coils. The MRI experiments were performed with a 4.7-T Varian Unity Inova MRI system (Varian Inc) equipped with shielded gradients. Diffusion-weighted images (DWI) and T2-weighted images (T2WI) were acquired. A diffusion-weighted spin-echo image was obtained by applying a motion-probing gradient of 0 and 4 g/cm. The β values were 0 and 420 s/mm2 for DWI. From these images, pixel-by-pixel maps of the apparent diffusion coefficient of water were calculated. Imaging parameters were as follows: 256×256 matrix; field of view, 60 mm; repetition time, 1200 ms; echo time, 60 ms; 2 average; 8 coronal slices; slice thickness, 1.5 mm; and gapless. The total imaging time was <15 minutes per image set. The entire rat brain was used to make a series of 1.5-mm contiguous coronal slices. MRI scans were generated and postprocessed with the use of MRVision (version 1.5.7; L.A. Systems Inc).

Histological Preparation
After MRI measurements, the rats were immediately euthanized. The brain was removed from the skull of each rat, fixed in 10% buffered formaldehyde solution for 2 days, and then cut into several 2-mm-thick coronal blocks using a brain slicer. All blocks were then embedded in paraffin. One 5-mm-thick section was taken at the center of the visible infarct and stained with hematoxylin and eosin to evaluate the infarct area.11

Immunohistochemistry
A primary rabbit polyclonal antibody to VEGF was used for VEGF staining. The streptavidin-biotin method was performed with a Histofine Max-PO kit (Nichirei Co). VEGF was visualized by using 300g/mL DAB. The sections were finally counterstained with hematoxylin.

Assessment of the Ischemic Damage in MR Images
We measured 3 types of ischemic lesions quantitatively using a microcomputer image analyzer: (1) a high-intensity area on DWI as CVI area; (2) a high-intensity area on apparent diffusion coefficient of water map as a vasogenic edema area; and (3) a high-intensity area on T2WI as an area that included both CVI and vasogenic edema. The experimenter performed data analysis without any previous information about the data. All different types of lesions were measured on every slice.

We estimated lesion volumes from MRI data. The area of ischemic damage in every slice appeared to resemble a “pudding.” The formula for the estimation of lesion volumes in each slice is as follows: A+B+(AB)1/2h/3, (A, B: lesion area [mm2] of each coronal section, h: height [distance of each section], mm).

The single lesion volumes were then added to estimate the entire ischemic damage. Volumes are compared statistically between the antagonist group and the PBS group.

Statistical Analysis
Data were expressed as means±SEM. Statistical significance was concluded at an error probability of P<0.05. All statistical comparisons were performed using Sigma-Stat software (Jandel Scientific).

Results
Physiological Variables
No significant differences were observed in physiological parameters (blood PaO2 and PaCO2, mean arterial blood pressure) before and after venous occlusion and between the groups.

Histological Findings
In all 25 rats, an infarction area had developed in the cortex. The ischemic damage of the PBS group was significantly larger than that of the antagonist group (0.95±0.26 mm2 versus 0.22±0.04 mm2; P<0.05) (Figures 1 and 2). Dilated spaces surrounding the capillaries around the infarction were prevalent in the PBS group, whereas in the antagonist group only a few dilated spaces surrounded capillaries around the infarction.

Immunohistochemistry
Brain sections incubated without the primary antibody did not take up the stain. In the presence of the primary antibody, immunoreactivity of VEGF was normally present in the ependymal cells (data not shown), whereas there was no
detectable immunoreactive VEGF in neurons, glial cells, vascular endothelial cells, or pial cells in noninjured brains.

One day after venous occlusion, immunoreactive VEGF was detected in the cytoplasm of a part of neurons in the cerebral cortex around the venous infarction in all animals (Figure 3).

**MRI Evaluation**

In the PBS group, DWI revealed cytotoxic edema, related to CVI, as high-intensity area on the left fronto-parietal cortex. Whereas, apparent diffusion coefficient of water map demonstrated vasogenic edema as a high-intensity area in the subcortical tissue around the CVI as observed in DWI. T2WI showed both CVI and vasogenic edema as a high-intensity area extending from the surface of cortex into the deep white matter. Midline shift was frequently observed because of swelling of the brain. These effects were less prominent in the antagonist group, which had smaller high intensity area on DWI and T2WI. Figure 4 shows typical examples of the MRI findings in both the groups.

**Correlation Between the Venous Infarct Area and a High-Intensity Area on DWI**

In case of this correlation, a high-intensity area on a DWI slice was assessed; this closely coincided with the location of the histological section. The correlation was highly significant ($r=0.96$, $P<0.01$, $n=25$) (Figure 5).

**Comparison Between the Antagonist Group and the PBS Group**

All calculated lesion volumes were significantly smaller in the antagonist group than in the PBS group: DWI (0.58±0.28 mm$^3$ versus 3.96±1.13 mm$^3$, $P<0.01$), ADC maps (0.71±0.45 mm$^3$ versus 2.80±0.75 mm$^3$, $P<0.05$), and T2W images (1.86±0.94 mm$^3$ versus 7.40±1.25 mm$^3$, $P<0.01$; Figure 6).

**Discussion**

Control of brain edema is the basic treatment that should follow brain injuries. Recent animal studies have shown that cytotoxic and vasogenic brain edema consistently occurs in acute CVI. CVI has received attention as a result of the increasing number of neurosurgical operations for aged patients and the development of skull base neurosurgery. However, the pathophysiology of cell death and edema formation remains unclear. The efficacy of heparin as a treatment been confirmed recently. Nevertheless, we use heparin with a lot of caution and, on many occasions, we only control brain edema because CVI often involves intracerebral hemorrhage, which might further affect brain edema development. We reported that parenchymal enhancement in MRI might predict the development of intracerebral hemorrhage in CVI. Mannitol is the most extensively studied agent with regard to reducing ischemic brain edema in animal mod-
els\textsuperscript{16–18} and patients.\textsuperscript{19,20} However, potential detrimental effects of mannitol have been noted.\textsuperscript{16,21} These may be related to osmotic reduction in water content in the normal brain rather than the ischemic brain, and dehydration may induce the decrease of cerebral perfusion pressure. Thus, mannitol has not been widely accepted in the management of ischemic brain edema, particularly in the CVI. We attempted to treat brain edema with a new treatment regimen to reduce CVI in a rat 2-vein occlusion (2-VO) model in this study.

In the rat 2-VO model, a widespread reduction, but not cessation of cortical blood flow has been observed. This characteristic cortical blood flow reduction led to a penumbra-like area and the development of small infarcts.\textsuperscript{11,24} Therefore, the pathophysiologic significance of the neurotoxic mediators induced/released by venous ischemia has been also studied in this model. For instance, induction of spreading depression worsens histological outcome after venous ischemia.\textsuperscript{25} This large low-flow area following cerebral venous ischemia results not only in angiogenesis but also in an increased VP; therefore, edema formation should be activated because of the induction of VEGF expression. Hence, we considered that the model was suitable for evaluating the relationship between VEGF and brain edema.

VEGF can markedly enhance angiogenesis in the ischemic brain\textsuperscript{26} and reduce neurological deficits during stroke recovery.\textsuperscript{27,28} These effects may be useful to prevent stroke in patients with repeated transient ischemic attacks.\textsuperscript{29} However, VEGF is also known as a potent VP factor. It has been reported that VEGF exacerbated VP and promoted ischemic cell damages.\textsuperscript{30}

In this study, we used a neutralizing antibody against VEGF, a rabbit polyclonal antibody, as VEGF antagonist. To check the possibility of the immunoreactions in the Wistar rat because of the exogenous immunoglobulin deduced from rabbit, we gave normal rabbit serum to 5 rats using the same procedure. However, we did not observe any immunoreaction in these animals and the ischemic damage was not significantly different from the PBS group (data not shown). The present study revealed that VEGF antagonist could reduce brain edema and CVI when VEGF antagonist was administered at the acute stage of 2-VO. This antagonist must affect through broken blood–brain barrier. Blood–brain barrier breakdown around the venous ischemia in this model has already been proven.\textsuperscript{23} Moreover, this antagonist must block at the receptor level, because VEGF expression was detected around the infarction even in the antagonist group. Josko et al stated that many VEGF inhibitors could be used in future for the prevention or treatment of hypoxia-induced cerebral edema.\textsuperscript{31} Efficacy of the inhibition of VEGF against VP has been reported in arterial ischemia model.\textsuperscript{8,32,33} Our findings might provide the basis for new therapeutic concepts for the treatment of brain edema after venous ischemia.

Figure 4. Representative MRI data for the 2 typical rats for each group (the PBS group, top row, number 7 and the Antagonist group, bottom row, number 20) are shown. The PBS group had large ischemic lesions (indicated by white arrow) and brain swelling (indicated by white dotted line). The ischemic area widely extends from the surface of cortex into white matter on the T2-weighted images (T2WIs). The venous infarction is detected in the cortex on the diffusion-weighted images (DWIs). The brain edema, particularly vasogenic edema, is detected around the infarction and widely spreads into the white matter on the apparent diffusion coefficient (ADC) of water map. The antagonist group had a smaller region than the PBS group in all types of images.

Figure 5. Correlation between the venous infarct area and a high intensity area on DWI. The position of a high-intensity area on DWI was coincident with venous infarction. The correlation of the size was significant ($r=0.96$, $P<0.01$, n=25).

Figure 6. Comparison of each ischemic lesion volume. The lesion volume was estimated using MRI data 24 hours after vein occlusion. The antagonist group had significantly smaller lesion volume than the PBS group in each ischemic lesion volume.
VEGF is an important angiogenesis factor, particularly in the hypoxia or ischemia-induced brain damage such as strokes. However, the positive effect of the inhibition of VEGF was shown by this experiment. This is supposed to be caused by the timing of the drug injection and the feature of CVI. VEGF antagonist was injected very early after occlusion and when compared with arterial ischemia, infarction matures slowly in the model. A balanced checkpoint should exist to determine the good and/or bad effect of VEGF in the stroke. Many neuroprotective agents in arterial ischemia have proven efficacious in animal models, but clinical trials with many agents have not given any promising results. One of the possible explanations is that the effects of neuroprotective agents on infarct size are time-dependent and treatment has often been initiated much later than in a successful experimental stroke models. CVI generally develops much slower than arterial stroke. Further, areas with moderate reduction of cortical blood flow causing a penumbra-like area surrounding a core infarct in CVI are wider than in arterial stroke. Therefore, therapeutic window in time and space is delayed and is much wider in CVI. Neuroprotective candidates with the success obtained with animal models are more promising in human trial in CVI than arterial infarct. Meanwhile, VEGF may be effective in the chronic stage in CVI. Therefore, we have to elucidate an appropriate manner and therapeutic time window of VEGF and anti-VEGF in stroke model.

**Conclusion**

We revealed that an inhibition of VEGF could reduce brain edema and CVI in the acute stage of CVI in a rat 2-VO model. This suggests that an inhibition of VEGF may be a new therapy for brain edema and CVI in the acute stage. However, VEGF is also an important angiogenesis factor. Thus, a balanced checkpoint should exist to determine the effect of VEGF in the stroke.

**References**

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