Kobe J. Med. Sci., Vol. 54, No. 2, pp. E136-E146, 2008

Anti-VEGF Receptor Antagonist (VGA1155) Reduces Infarction in Rat Permanent Focal Brain Ischemia

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Received 6 February 2008/ Accepted 3 March 2008

Key words: VEGF (vascular endothelial growth factor), cerebral ischemia, cerebral Infarction

Background and Purpose: Vascular endothelial growth factor (VEGF) is a major mediator of angiogenesis and a strong vascular permeability factor. VEGF is known to open the blood-brain barrier (BBB) and induce cerebral edema. Experimental studies of VEGF antagonism have shown it reduces cerebral edema after ischemia, indicating its potential for prevention of secondary brain damage. We examined the therapeutic effect of VGA1155 (5- [N-Methyl-N- (4-octadecyloxyphenyl) acetyl] amino-2-methyl-thiobenzoic acid), a novel small molecule antagonist of VEGF, on rat permanent focal cerebral ischemia.

Methods: Permanent middle cerebral artery occlusion (MCAO) was induced with the suture occlusion method. A single dose of VGA1155 (10mg/kg, i.p.) was administered 30 minutes before the induction of MCAO after which brain water content, Evans blue extravasation, neurological score, infarct volumes and VEGF expression determined by means of ELISA were compared with corresponding values for vehicle injected control rats.

Results: Brain water content and Evans Blue extravasation 24 hours after ischemia were not significantly reduced, but, compared with control group, VGA1155 significantly reduced infarct volume (32.0% for VGA1155 vs. 46.7% for control; % volume of hemisphere volume) and improved neurological function 7 days after ischemia, when tissue content of the VEGF group markedly increased to nine times that of the vehicle-treated animals.

Conclusion: VGA1155 was found to protect against secondary ischemic brain damage after permanent focal cerebral ischemia, although it did not reduce vasogenic edema at 24 hours. Changes in endogenous VEGF may be related to the therapeutic effect of VGA1155.

Brain edema has been classified into two types: cytotoxic and vasogenic (26).Cytotoxic edema is a premorbid process that involves oncotic swelling of cells accompanied by the movement of osmotically active molecules from the extracellular to the intracellular space. Vasogenic edema is caused by disruption of the blood-brain barrier (BBB) induced by various forms of brain damage, such as cerebral ischemia, encephalitis and brain. Vascular endothelial growth factor (VEGF) is a vascular permeability factor and a mitogen associated

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with angiogenesis (3,8,9). VEGF is known to be rapidly induced after focal cerebral ischemia (11) and to bind to two tyrosine kinase receptors, fms-like tyrosine kinase-1 (Flt-1) and the kinase insert domain containing receptor/fetal liver kinase-1 (KDR/Flk-1), on the surface of endothelial cells (9). However, the biological effect of VEGF on BBB in the ischemic brain remains unknown (31). Early administration of recombinant VEGF to ischemic rats was found to significantly increase BBB leakage and brain edema (32), suggesting that suppression of VEGF is beneficial for secondary brain damage due to edema after focal brain ischemia. A recent report described the protective effects of gene transfer of soluble Flt-1 (sFlt-1), a natural inhibitor of VEGF, on focal cerebral ischemia (19).

Various agents of VEGF antagonists have been developed, e.g., anti-VEGF antibody (15,17), anti KDR/Flk-1 antibody (24), 2'-fluoropyrimidine RNA-based aptamers (25), various peptides (2,5), porphyrin analogues (1) and sFlt-1 (22). van Bruggen reported that administration of mFlt (1-3) -IgG in murine cerebral ischemia significantly reduced the volume of edematous tissue (30). Synthesis of some of these antagonists, however, is costly because of their large molecular weight. The antibodies and peptides also have been found to carry the risk of antigenicity and instability *in vivo*. For clinical application, therefore, a lower molecular weight antagonist would be more suitable. VGA1155 (5-[N-Methyl-N-(4-octadecyloxyphenyl) acetyl] amino-2-methylthiobenzoic acid) is a novel antagonist of VEGF with a small molecular weight of 583.87. By binding to the VEGF receptors Flt-1 and Flk-1, VGA1155 reportedly inhibits their VEGF-induced autophosphorylation (28). VGA1155 is known to have low toxicity and to have little effect on other growth factors and cytokines (29). Moreover, we recently showed that VGA1155 reduces vascular permeability, cerebral edema and cerebral infarction in rat transient focal cerebral ischemia (under submission). The aim of the study presented here was to determine the therapeutic effect of VGA1155 on rat permanent focal ischemia.

MATERIALS AND METHODS

Experimental animals and groups. All procedures involving animals were approved by the Animal Care and Use Review Committee of Kobe University Graduate School of Medicine. Young adult male Sprague Dawley rats weighing 280-380g (Clea Japan, Inc.; Osaka, Japan) were used for this study. The rats were housed in a controlled environment (alternating 12-hour light/dark cycle, 22±2℃, 55±5% relative humidity) and allowed free access to food and tap water throughout the experiments.

The 65 rats used for this study were randomly divided into three groups in a blinded manner. The first group was sham operated (n=5), while the second group was vehicle injected after ischemia, with phosphate buffer intraperitoneally injected 30 minutes before the onset of cerebral ischemia (n=30). The third group was treated with VGA1155 at a dosage of 10mg/kg administered intraperitoneally 30 minutes before permanent cerebral ischemia (n=30).

VGA1155 was synthesized and kindly provided by the Medicinal Pharmacology Laboratory of Taisho Pharmaceutical Co., Ltd., Saitama, Japan. For this experiment, VGA1155 was dissolved (4mg/ml) in isotonic phosphate buffer (pH9.0).

Middle cerebral artery occlusion (MCAO). Rats were anesthetized with halothane and maintained at 1.5% halothane in 70% nitrous oxide and 30% oxygen, which allowed them to breathe spontaneously. Rectal temperature was maintained at 37.3℃±0.5℃ with a feedback-regulated heating pad during the procedure. The right femoral artery was cannulated to monitor arterial blood pressure by means of a pressure transducer (AP-601G; Nihon Koden Inc., Tokyo, Japan) and a data acquisition system (UAS-108S; Unique Medical

Inc., Tokyo, Japan) and to obtain blood samples before and after ischemia for analysis of blood gas, electrolytes and blood glucose by means of a blood gas analyzer (iSTAT®). For monitoring changes in regional cerebral blood flow (rCBF) of the right hemisphere, a thin probe (TBF-LN1; Unique Medical) used for Laser-Doppler flowmetry was placed between the right temporal muscle and the right temporal bone. Permanent focal cerebral ischemia was induced with the suture occlusion technique. Briefly, the right common carotid artery (CCA), internal carotid artery (ICA) and external carotid artery (ECA) were exposed through a ventral cervical midline incision, after which the pterygopalatine artery, ECA and CCA were ligated with a 7-0 silk suture. The ICA was then closed with a microvascular clip and a 7-0 silk suture was tied loosely around the CCA. A small incision was made in the CCA, and a 4-0 monofilament nylon suture coated with silicone was introduced into the ICA through the CCA until the laser-Doppler signal showed a steep drop. Finally, a 7-0 silk suture was tightened around the CCA and the intraluminal thread, anesthesia was discontinued, and the animals were allowed to recover. Animals with a reduction in the rCBF above 30% of the base line value or having died of subarachnoid hemorrhage were excluded from the experimental protocol (31 rats in all). Sham-operated rats underwent surgery without the filament insertion.

Physiological parameters were checked randomly in rats injected intraperitoneally with VGA1155 (10mg/kg) or vehicle (n=5 for either group). Arterial blood pressure, arterial blood gas, blood pH, blood glucose concentration and plasma electrolytes, were measured before and after occlusion of the MCA. There were no significant differences between the VGA1155-treated group and the vehicle group at any time point (Table I).

Table I. Physiological Parameters

	Blood pressure (mmHg)	PCO2 (mmHg)	PO2 (mmHg)	pH	Glu (mg/dl)
Vehicle					
Before ischemia	93.4 ± 7.0	43.4 ± 3.3	112.0 ± 7.48	7.39 ± 0.01	128.4 ± 13.0
During ischemia	95.8 ± 2.8	46.4 ± 1.9	139.0 ± 14.5	7.43 ± 0.01	132.8 ± 13.8
VGA1155 treated (10mg/kg)					
Before ischemia	100.7 ± 7.4	43.9 ± 2.1	113.8 ± 5.12	7.39 ± 0.01	125.0 ± 13.1
During ischemia	95.5 ± 5.5	45.1 ± 2.3	131.8 ± 7.60	7.44 ± 0.02	132.0 ± 16.5

Values are expressed as means as ± S.D. (n=5 for each group). There were no significant differences in mean arterial blood pressure, arterial gases or blood glucose concentration among the groups.

Thirty-five rats were sacrificed 24 hours postoperatively for measurement of water content (n=5 from each subgroup), blood-brain-barrier permeability (n=5 from each subgroup) and cerebral infarction volume (n=5 from each subgroup). Twenty rats were sacrificed 4 days (n=5 from each subgroup) and 7 days (n=5 from each subgroup) after the onset of cerebral ischemia for evaluation of VEGF expression by means ELISA. Ten rats were sacrificed 7 days (n=5 from each subgroup) after MCAO for long-term evaluation.

Measurement of brain water content. Fifteen rats (n=5 from each main group) were sacrificed by decapitation under deep anesthesia 24 hours after cerebral ischemia. The brains were removed and divided into hemispheres, each of which was weighed to obtain the wet weight and then dried at 110℃ for 24 hours. The water content in the hemisphere was calculated as water content (%) = (wet weight – dry weight) / wet weight × 100.

Blood-brain barrier permeability for Evans blue. Ten rats (n=5 each from the vehicle-injected and the permanent ischemia treated with VGA1155 group) were given 2ml/kg of 2% Evans Blue solution intravenously 22 hours after induction of ischemia. Two hours after the dye injection, the rats were re-anesthetized by intraperitoneal injection of 50 mg/kg sodium pentobarbital and perfused with saline through the left ventricle at 90 mmHg pressure until colorless perfusion fluid was obtained from the right atrium. After decapitation of the rats, the brains excluding the cerebellum were removed rapidly from the decapitated rats and divided into hemispheres. Each hemisphere was weighed, homogenized in 2 ml of 50% trichloroacetic acid (wt/vol), and centrifuged at 10,000 rpm for 20 minutes. The extracted dye was then diluted with ethanol (1:3), and its fluorescence was determined (excitation at 620 nm and emission at 680 nm) with a luminescence spectrophotometer. Calculations were based on the external standard (62.5-500ng/ml) in the same solvent. Evans Blue contained in tissue was quantified from a linear standard line derived from known amounts of the dye and was expressed in terms of Evans Blue (μg) / tissue (g)

Neurological assessment. Ten rats (n=5 from the vehicle-injected and n=5 from the VGA1155-treated group) were subjected to a battery of neurological tests before induction of cerebral ischemia as well as 1 and 7 days after MCAO. The established neurological scoring system (4) was used for these tests. Each rat was scored with a discrete value of 0 (no apparent deficits), 1 (contralateral forelimb flexion), 2 (decreased grip of the contralateral forelimb while animal is pulled by tail), 3 (spontaneous movement in all directions; contralateral circling only if animal is pulled by tail), 4 (spontaneous contralateral circling) or 5 (death).

Quantification of infarct volume. Twenty rats (n=5 from each subgroup) were anesthetized 24 hours after cerebral ischemia by intraperitoneal injection of 50 mg/kg sodium pentobarbital and their brains were removed. Seven coronal slices made from the frontal pole were immersed into a 2 % solution of triphenyltetrazolium chloride (TTC) in normal saline at 37℃ for 30 minutes, and were then fixed in 10 % phosphate-buffered formalin. Seven days after cerebral ischemia, another 20 rats (n=5 from each subgroup) were anesthetized by intraperitoneal injection of 50 mg/kg sodium pentobarbital and transcardially perfused with normal saline and then with 4 % paraformaldehyde in 0.1 mol/L sodium phosphate, pH7.4. Brains were carefully removed, fixed in 4 % paraformaldehyde solution and processed for paraffin embedding. The brains thus embedded were then sectioned into 5μn thicknesses at 0.5mm intervals from 1mm anterior to 3.5mm posterior to the bregma and stained with hematoxylin and eosin (HE). For each study, the contralateral hemisphere area, the ipsilateral non-infarct area and the total brain area were measured by using Image Pro Plus Ver. 5.0 (Media Cybernetics, Inc., Silver Spring, MD) and the areas were multiplied by the distance between sections to obtain the respective volumes. Infarct volume was

calculated as a percentage of the volume of the contralateral hemisphere with the following formula: infarct volume = [volume of contralateral hemisphere – ipsilateral intact volume] / volume of contralateral hemisphere × 100.

Quantification of VEGF expression. Four days and seven days after cerebral ischemia, 20 rats were anesthetized by intraperitoneal injection of 50 mg/kg sodium pentobarbital and perfused with saline through the left ventricle at 90 mmHg pressure until colorless perfusion fluid was obtained from the right atrium. After the rats had been decapitated, their brains, except for the cerebellum, were removed rapidly and separated into hemispheres. Ipsi-hemispheres were then sectioned into 2mm thicknesses at the midpoint of the coronal section and divided into medial and lateral pieces. The medial pieces were homogenized in 1 ml of 1×PBS and centrifuged at 10,000 rpm for 20 minutes. The supernatants were diluted for adjustment of the protein concentration. Protein concentrations of the supernatants were determined with the Bradford method using BSA as the standard. VEGF was then quantitated with a commercially available VEGF ELISA kit (Quantikine M; R&D systems, Minneapolis, MN) according to the manufacturer's instructions.

Statistical analysis. All values are expressed as means ± standard deviation. Statistical analysis was carried out with unpaired Student's *t* test and one-way ANOVA. P values less than 0.05 were considered statistically significant.

RESULTS

Water content of the ipsilateral cerebral hemisphere had increased significantly 24 hours postoperatively in the vehicle-injected (80.8 ± 0.5%) and VGA1155-treated ischemic rats (80.9 ± 0.4%) compared with the sham-operated rats (75.9 ± 0.1%, $P<0.01$) (Fig. 1-a). VGA 1155 did not reduce edema formation after 24 hours.

Figure 1-a

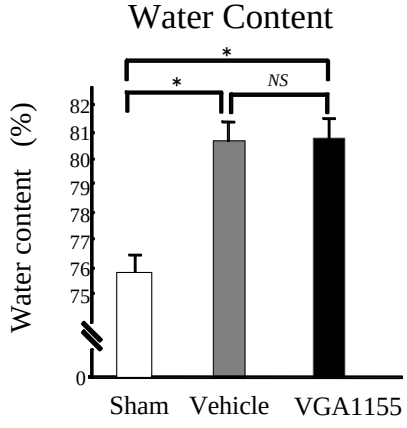

Figure 1-b

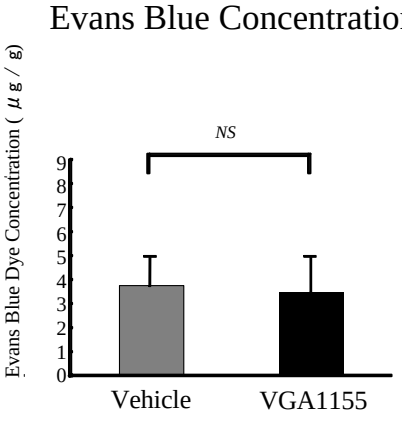

Figure 1. (a) Water content measured 24 hours after ischemia. The following calculation was used: water content (%) = (wet weight – dry weight) / wet weight × 100. Values are expressed as means ± S.D. (n=5 for vehicle-injected and n=5 for VGA1155-treated). * $P<0.01$. NS: not significant.
(b) Vascular permeability in terms of Evans Blue concentration 24 hours after ischemia. Values are expressed as means ± S.D. (n=5 for vehicle-injected and n=5 for VGA1155-treated).

Extravasation of Evans blue during two hours following injection was 3.8±1.2 μg/g of wet tissue in the vehicle-injected ischemic rats and 3.5±1.5 μg/g in the VGA1155-treated ischemic rats, which was not statistically significant (Fig. 1-b). Increased permeability after 24 hours was not affected by VGA 1155.

On day 1 after the onset of ischemia, there was no difference in neurological scores between vehicle-injected rats (2.86 ± 0.4) and rats treated with VGA1155 (2.86 ± 0.7) (Fig. 2). On day 7, rats treated with VGA1155 had significantly improved compared with control animals (0.7 ± 0.5 vs. 1.6 ± 0.7, $P< 0.01$) (Fig. 2).

Figure 2

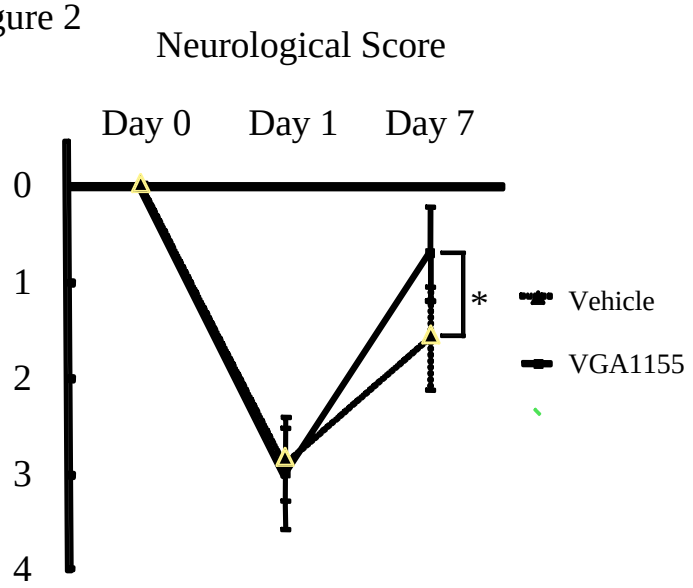

Figure 2. Time course of neurological score before induction of cerebral ischemia and on days 1 and 7 after middle cerebral artery occlusion (MCAO). Values are expressed as means ± S.D. (n=5 for both vehicle-injected and VGA1155-treated group). * $P<0.01$.

One day after the onset of ischemia, there were no significant differences in infarct volume between the control group (51.6 ± 4.5%) and VGA1155-treated group (51.2 ± 6.7%). Seven days after ischemia, however, the infarct volume of the VGA1155-treated rats was significantly smaller than that of the control group (32.0 ± 4.4% vs. 46.7 ± 2.1%) (Fig. 3a-d).

In the vehicle-injected control rats, VEGF on day 4 after ischemia was 3.5 ± 4.0 pg/ml and declined to 0.5 ± 4.0 pg/ml on day 7. In the VGA1155-treated rats, on the other hand, VEGF was significantly lower on day 4 (1.0 ± 3.0 pg/m), but markedly higher on day 7 (4.5 ± 4.0pg/ml) in comparison with the control (Fig. 4). Seven days after permanent MCAO had been established, the increase in VEGF expression of the VGA1155-treated group was nine times that in the vehicle control group.

Figure

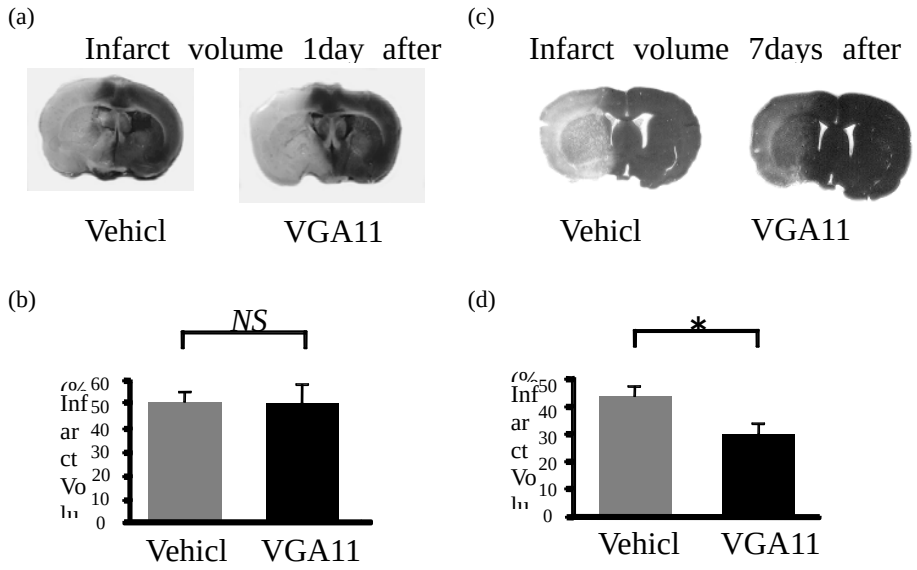

Figure 3. Quantification of infarct volume on days 1 and 7 after MCAO. The upper sections show TTC-staining on day 1 (a) and hematoxylin and eosin staining on day 7 (c). There was no significant difference between the vehicle-injected and VGA1155-treated group (b). Seven days after MCAO, VGA1155-treated rats showed less infarct volume than their vehicle-injected counterparts (d). Values are expressed as means ± S.D. (n=5 for either group). * P<0.01.

Figure 4

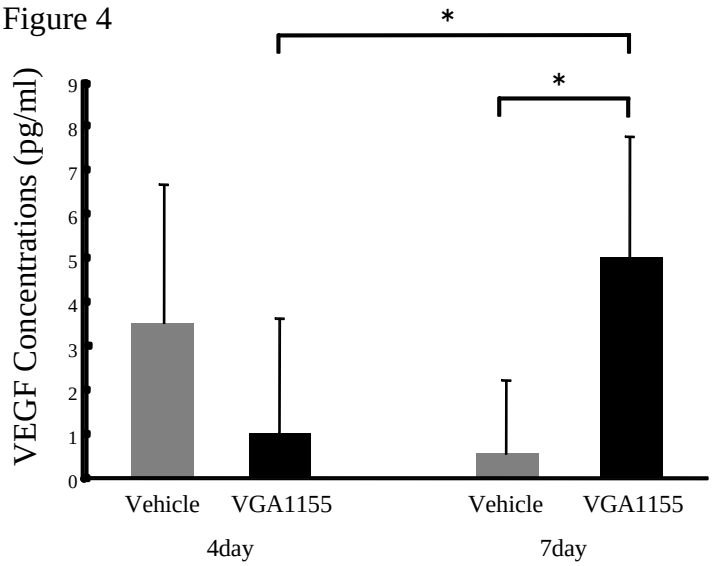

Figure 4. Quantification of VEGF expression of ischemic lesion on days 4 and 7 days after ischemia analyzed by ELISA. Values are expressed as means ± S.D. (n=5 for either group). * P<0.05.

DISCUSSION

This study demonstrated the beneficial effect of VGA1155 in cerebral ischemia, since in the permanent MCAO model rat, the infarction volume on day 7 after the onset of ischemia was histologically reduced from 46.7% to 32.0% of the intact hemisphere. Furthermore, behavior impairment after ischemia had markedly diminished at 7 days (Fig. 2). We first thought that this beneficial effect could be the result of the reduction of brain edema in the early phase. In the literature (6,16,30), studies using various antagonists to VEGF have provided evidence of a reduction of brain edema followed by a decrease in the infarction tissue volume. We also studied the impact of VGA1155, an antagonist of VEGF, on focal reperfusion cerebral ischemia in a manner similar to that used for the present study and obtained a significant reduction of tissue water content from 81.8% to 80.2% ($P<0.05$) on day 1 postischemia (under submission). In addition to VGA1155, the intraperitoneal injection was used for another VEGF antagonist or a fusion protein inhibiting Flt-1-related receptors (30), as well as topical application of a VEGF antibody (12). Regardless of the agent, the reduction in brain edema in the early phase of postischemia, between 24 - 48 hours after the onset when endogenous VEGF is being upregulated, was considered to be the most likely main path for the development of VEGF antagonism.

However, our present study demonstrated that edema formation was not affected by VGA1155 on day 1 postischemia. This was thus an unexpected finding and contradicted the results of previous studies. There are several possible explanations for this absence of brain edema reduction. First, the permanent MCAO model used in our study featured total proximal occlusion, which is associated with severer hemodynamic disruption than the occlusion used in any of the previous studies (17,10,30). Moreover, intraperitoneally administrated VGA1155 may have been ineffectively delivered to the ischemic core, and this may account for a distinct difference from the reperfusion ischemic models reported in our previous literature (under submission). Second, most of the previous studies (13,14,30) used MR images for evaluating brain edema but we used water content and permeability of dye. For MR images, brain edema was defined by the regions of T2 hyperintensity and measured as the extent or area of the region, whereas the water tissue content method basically compares the weight of whole hemispheres for a quantitative measurement of brain edema. Radiological imaging is probably more sensitive than measuring the water content for detecting change in edema. However this may be, brain edema reduction using VGA1155 on day 1 postischemia in permanent MCAO was very minor, if any at all, and much less than that in the reperfusion MCAO established in our laboratory. Failure to rescue the ischemic lesion on day 1 as demonstrated by TTC staining was thus in agreement with insignificant reduction of brain edema.

In our study, VEGF at 7 days after ischemia in the VGA1155-treated group was significantly higher than in the vehicle-administered group. Under conditions of ischemia, VEGF mRNA expression is known to be induced by hypoxia (21). Previous studies (20,23) have demonstrated that the increase in VEGF during cerebral ischemia is relatively short, with VEGF mRNA levels increasing 12 - 48 hours after transient cerebral ischemia and then returning to basal expression after 7 days. VEGF protein was detected in neurons from 18 hours after the onset of ischemia until day 5 in permanently ischemic rats (18). The prolonged high level of brain VEGF induced by VGA1155 as observed in our study is therefore of interest from the viewpoint of endogenous VEGF signaling and metabolites. One can be speculated that a single injection of VGA1155 before cerebral ischemia resulted in prolonged activation of VEGF generation or inhibition of VEGF degradation, but further studies are necessary to identify the exact mechanisms involved in this activation.

TTC staining, performed on day 1, showed mitochondria energy failure, and HE staining, performed on day 7, demonstrated infracted tissue necrosis. It is clear that VGA1155 rescued the penumbra tissue where the secondary process of brain damage from energy failure to neuronal cell death was proceeding. Sun et al. (27) showed that in the ischemic brain VEGF exerts an acute neuroprotective effect, as well as longer latency effects on survival of neurons and on angiogenesis. In addition, Hayashi et al. (12) applied VEGF topically to the surface of the brain after focal ischemia and found that infarct volume was reduced. From the viewpoint of molecular mechanism, VEGF regulates the cell survival though the phosphophatidylinositol 3' kinase /Akt signal transduction pathway (7). In our study, promotion of neurogenesis and angiogenesis activated by prolonged high levels of endogenous VEGF likely play a partial role to reduce infarct volume. From a clinical viewpoint, however, an inadequate increase in endogenous VEGF involves a risk of induced deleterious brain edema. It is unclear, however, whether a single injection just prior to ischemia, as used in our study, is the best method to administer VGA1155. The half-life of VGA1155 in nude rat plasma was relatively long, about 21 hours, when it was given intraperitoneally at a dose of 10 mg/kg (data not shown). However, further studies should be performed to evaluate the effect of changing the timing of VGA1155 administration.

Several kinds of VEGF antagonists have been developed, including anti-VEGF antibody (15, 18), anti KDR/Flk-1 antibody (24), 2'-fluoropyrmidine RNA-based aptamers (25), various peptides (2, 5), porphyrin analogues (1), mFlt1 (1-3)-IgG (30) and soluble Flt-1 (22). While clinical trials using several VEGF inhibitors for cancer patients are ongoing, hardly any basic or clinical research of VEGF inhibitors for brain edema or brain ischemia is being performed. We believe that such research is of vital importance since our findings indicate that targeting the VEGF system by using VGA1155 is a valid approach to the treatment of cerebral ischemia and stroke.

ACKNOWLEDGEMENTS

We are grateful to the Taisho Pharmaceutical Company, Japan, for providing the VGA1155. This study was supported by a grant-in-aid for Scientific Research (B)(2)(16390410) to E.K., (C)(2)(17591513) to T.K. and (B)(17790968) to T.S. from the Ministry of Education, Science and Culture of Japan. We deeply appreciate A. Kato, for technical assistance.

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