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The Novel VEGF Receptor Antagonist, VGA1155, Reduces Edema, Decreases Infarct and Improves Neurological Function after Stroke in Rats

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Vascular endothelial growth factor (VEGF) is a major mediator of angiogenesis and also a strong vascular permeability factor. Blockade of VEGF may have a potential to treat cerebral edema after brain injury. We evaluated the effect of VGA1155 (5-[N-Methyl-N-(4-octadecyloxyphenyl)acetyl]amino-2- methylthiobenzoic acid), a novel binding antagonist of VEGF, on cerebral edema after transient focal cerebral ischemia. Focal cerebral ischemia was induced with the suture occlusion method for 2 h. In the treatment group, a single dose of VGA1155 (1 ~ 50 mg/kg i.p.) was administrated 30 min before the induction of focal ischemia, and the vehicle group received phosphate buffer only. The brain water content, Evans blue extravasation, infarct volumes and neurological score were determined. Physiological parameters were not influenced by the administration of VGA1155. The brain water content at 24 h after cerebral ischemia was significantly reduced by intraperitoneal administration of VGA1155 and the dose of 10 mg/kg showed the maximum effect on brain water content (81.8 ± 0.5% in non treated group vs. 80.2 ± 0.6% in treated group). With this dose, VGA1155 also reduced vascular permeability from 2.2 ± 0.8 μg/g to 1.2 ± 0.5 μg/g studied at 6 h after the ischemia by intravenous injection of Evans blue. VGA1155 administration significantly reduced infarct volume and improved neurological scores at 1 week after ischemic injury. The data suggested that VGA1155 has antiedematous effect in acute phase after transient focal cerebral ischemia and improves neurological and histological outcomes 1 week after ischemic injury.

VEGF is known as the major inducer of angiogenesis and also increases vascular permeability [3, 6, 7]. Rapid induction of VEGF after transient focal cerebral ischemia has been demonstrated [10]. In acute phase of cerebral ischemia, endogenous VEGF can be involved in the development of cerebral edema, increasing vascular permeability. Early administration of VEGF increases blood-brain barrier (BBB) permeability and deteriorates cerebral edema in cerebral ischemia [28]. VEGF antagonism has been considered to be beneficial for cerebral edema after cerebral ischemia [28]. Previously, antagonism of VEGF by a mFlt1(1-3)-IgG has been shown to reduce edema formation and tissue damage after transient focal cerebral ischemia [26]. Protective effects of gene transfer of soluble Flt-1 (sFlt-1), a natural inhibitor of VEGF, on focal cerebral ischemia has been reported [14]. It

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has been proposed that members of the Src family mediate VEGF-dependent vascular permeability. It has been reported that Src-/- mice are resistant to VEGF-induced vascular permeability and show decreased infarct volumes after stroke [19].

There have been several VEGF antagonist, including anti-VEGF antibody [11, 12], anti KDR/Flk-1 antibody [20], 2'-fluoropyrimidine RNA-based aptamers [21], various peptides [2, 5], porphyrin analogues [1] and soluble Flt-1 [18]. However, these antagonists may demand high cost of synthesis because of their large molecule weight. Furthermore, there is concern that antibodies and peptides may have antigenecity and instability in vivo. In clinical practice, a low molecular weight antagonist may be ideal. VGA1155, a novel low molecular weight antagonist of VEGF, inhibits binding between VEGF and its receptors, Flt-1 and KDR/Flk-1. It is known that VGA1155 shows lower toxicity and has little effect on other growth factors and cytokines [24, 25]. We have recently shown that inhibition of VEGF by VGA1155 reduces vascular permeability and cerebral edema in the rat cold injury model [13]. The aim of this study is to define therapeutic effect of VGA1155 on cerebral edema in the rat focal cerebral ischemia model.

MATERIALS AND METHODS

Animals

The experiments were conducted according to the Guidelines for Animal Experiments of the Kobe University School of Medicine. Male Sprague-Dawley rats weighing 280-310 g (Clea Japan, Inc.; Osaka) were used for this study. The animals were housed in a controlled environment (alternating 12-hour light/dark cycle, 22 ± 2°C, 55 ± 5% relative humidity) and allowed free access to food and tap water throughout the experiments.

Middle cerebral artery occlusion (MCAO)

Rats were anesthetized with halothane and maintained at 1.5% halothane in 70% nitrous oxide and 30% oxygen allowing to breath spontaneously. Rectal temperature was maintained at 37.5 ± 0.5℃ with a feedback-regulated heating pad during the operation. The right femoral artery was cannulated to monitor arterial blood pressure using a pressure transducer (AP-601G; Nihon Koden, Tokyo, Japan) and a data acquisition system (UAS-108S; Unique Medical Inc., Tokyo, Japan) and to obtain blood sample for evaluate blood gas, electrolytes and blood glucose using a blood gas analyzer (iSTAT®). For monitoring the change of rCBF of the right hemisphere, a thin probe (TBF-LN1; Unique Medical Inc., Tokyo, Japan) of the laser-Doppler flowmetry was placed between the right temporal muscle and the right temporal bone [8]. Transient focal cerebral ischemia was induced by the suture occlusion technique [15]. In brief, the right common carotid artery (CCA), internal carotid artery (ICA) and external carotid artery (ECA) were exposed through a ventral cervical midline incision. The pterygopalatine artery, ECA and CCA were ligated with a 7-0 silk suture. The ICA was 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 decrease. A 7-0 silk suture was tightened around the CCA and the intraluminal thread. Anesthesia was then discontinued, and the animals were allowed to recover. At 2 hours after MCAO, the occluding filament was withdrawn from the CCA under brief halothane anesthesia. We made sure of the successful reperfusion with the laser-Doppler flowmetry. We used a criteria to obtain consistent ischemic injury using laser Doppler flowmetry as follows; a) more than 30% reduction of rCBF is necessary for successful induction of ischemia, b)

more than 2-fold rapid increase in rCBF is necessary for complete reperfusion. Sham-operated rats underwent identical surgery except the filament insertion.

Drug administration

VGA1155 (5-[N-Methyl-N-(4-octadecyloxyphenyl) acetyl] amino-2-methylthiobenzoic acid) was synthesized in Taisho Pharmaceutical Co., Ltd., Saitama, Japan and kindly provided. For this experiment, VGA1155 was dissolved in isotonic phosphate buffer (pH 9.0) and administered by intraperitoneal (i.p.) injection.

Forty-five rats were randomly divided into five groups in a blinded manner. In the control group (n=15), vehicle phosphate buffer (pH9.0) was used at thirty minutes before cerebral ischemia. In dose response study, between 1 and 50 mg / kg i.p. was chosen because over 100 mg / kg results in fetal side effect of bleeding in spleen and kidney (personal communication). VGA 1155, at the dosages of 1 mg / kg (n = 5), 10 mg / kg (n = 15), 25 mg / kg (n = 5) and 50 mg / kg (n = 5) was administered at thirty minutes before MCA occlusion. Thirty animals were sacrificed at 24 hours for measurement of brain water content (n = 5 for each subgroup), and ten animals at 6 hours for blood-brain barrier permeability (n = 5 for 10 mg / kg group and untreated group). Ten animals were sacrificed at 7 days after cerebral ischemia for long-term evaluations.

Measurement of brain water content

The rats were sacrificed by decapitation under deep anesthesia at 24 hours after cerebral ischemia. The brains were removed rapidly and divided into hemispheres. Each hemisphere was weighed to obtain the wet weight and dried at 110℃ for 24 hours. The water content in the hemisphere was calculated as follows: water content (%) = (wet weight - dry weight) / wet weight × 100.

Blood-brain barrier permeability to Evans blue

2 ml/kg of 2% Evans Blue solution was administered intravenously at just after the reperfusion. At 6 hours after cerebral ischemia, the animals were reanesthetized 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 and separated in each hemisphere. 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 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 (62.5-500 ng / ml) in the same solvent. The tissue content of Evans Blue 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

A battery of neurological evaluations were performed before cerebral ischemia and at 1 and 7 days after cerebral ischemia in the control group and the VGA1155-treated group (10 mg / kg), using the established neurological scoring system [4, 17]. Each rat was given a discrete value of 0 (no apparent deficits), 1 (contralat forelimb flexion), 2 (decreased grip of the contralat forelimb while animal is pulled by tail), 3 (spontaneous movement in all

directions; contralat circling only if animal is pulled by tail), 4 (spontaneous contralat circling) or 5 (death).

Quantification of infarct volume

Seven days after cerebral ischemia, rats (n = 5 for 10 mg / kg treated group and untreated control group) were anesthetized by intraperitoneal injection of 50 mg / kg sodium pentobarbital and transcardially perfused with normal saline and then 4% paraformaldehyde in 0.1 mol/L sodium phosphate, pH7.4. Brains were carefully removed, fixed in 4% paraformaldehyde solution and immersed in phosphate-buffered 30% sucrose. Paraffin-embedded brains were then sectioned in 5μm thickness at 0.5 mm intervals from 1 mm anterior to 3.5 mm posterior to the bregma and stained with hematoxylin and eosin. Contralateral hemisphere area, ipsilateral non-infarct area and total brain areas were measured using Image Pro Plus Ver. 5.0 (Media Cybernetics, Inc) and 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.

Statistical analysis

All values are expressed as means ± standard deviation. Physiological parameters and water content were analyzed by one-way ANOVA followed by post hoc comparison with the use of Bonferroni/Dunn test. Evans blue extravasation, infarct volumes and neurological scores were analyzed by using unpaired Student's t test. Statistical significance was accepted at $P < 0.05$.

RESULTS

Physiological parameter

There were no significant differences of mean arterial blood pressure, arterial blood gas, blood pH, blood glucose concentration, plasma electrolytes or osmolality among the experimental groups at each time point (Table I).

Table I. Physiological Parameters

	Blood pressure (mm Hg)	PCO_2 (mm Hg)	PO_2 (mm Hg)	pH	Glu (mg/dl)
Vehicle					
Before ischemia	98.8±2.5	42.0±4.6	103.0±20.2	7.44±0.04	170.8±20.6
During ischemia	91.4±13.0	42.1±3.9	102.8±14.0	7.41±0.03	150.2±29.6
VGA1155-treated (10mg/kg) at 0.5 h before MCAO					
Before ischemia	98.5±4.3	42.1±3.8	95.6±19.2	7.43±0.03	164.0±29.3
During ischemia	96.2±10.7	40.1±3.2	95.4±7.8	7.42±0.02	143.6±14.4
VGA1155-treated (25mg/kg) at 0.5 h before MCAO					
Before ischemia	97.2±3.4	42.3±3.0	94.8±15.8	7.44±0.04	174.2±14.2
During ischemia	97.0±13.0	40.1±2.2	98.6±16.5	7.41±0.02	148.0±13.5

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

Brain water content

The water content of the ipsilateral hemisphere was significantly increased after transient focal cerebral ischemia in 24 hours to 81.8 ± 0.5 % (n = 5), compared to that of the sham-operated animal (78.6 ± 0.1 % (n = 5), P < 0.01, Figure 1.). The effect of VGA1155 given 30 minutes before focal cerebral ischemia are presented in Figure 1. A significant reduction of water content was achieved by two doses of 10 and 25 mg / kg (80.2 ± 0.6 % (n = 5), 80.5 ± 0.7 % (n = 5), P < 0.05 vs. vehicle controls, Figure 1.), respectively. An additional increase in the dose to 50 mg / kg did not result in a significant decrease in water content.

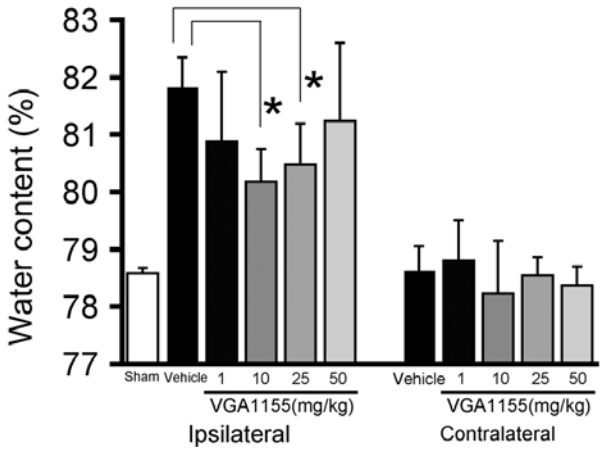


Figure 1. Water content measured 24 hours after MCA occlusion. Values are expressed as means ± S.D. (n = 5, in each group). The ipsilateral water content after transient focal cerebral ischemia was significantly increased to 81.8 ± 0.5 %, compared to that of the sham-operated animal (**P < 0.01). In dose-effect studies, VGA1155 doses of 10 and 25 mg / kg given 30 minutes before MCA occlusion significantly attenuated the increase of water content (* P < 0.05).

Blood-brain barrier permeability to Evans blue

The degree of blood-brain barrier disruption, as indicated by the extravasation of Evans blue during the 4 hours reperfusion, was 2.2 ± 0.8 μg / g of wet tissue (n = 5, Fig. 2). ; the ischemic hemispheres of the treated group with 10 mg / kg of VGA1155 showed 45 % reduction of extravasated Evans Blue in comparison to rhose of non-treated vehicle injected control group. (1.2 ± 0.1 μg / g (n = 5), P < 0.05, Figure 2.). ; The maximum effect to reduce edema was found at 10 mg / kg. 25 to 50 mg / kg showed less effects suggesting some toxicity at higher doses that may be related to bleeding at 100 mg / kg.

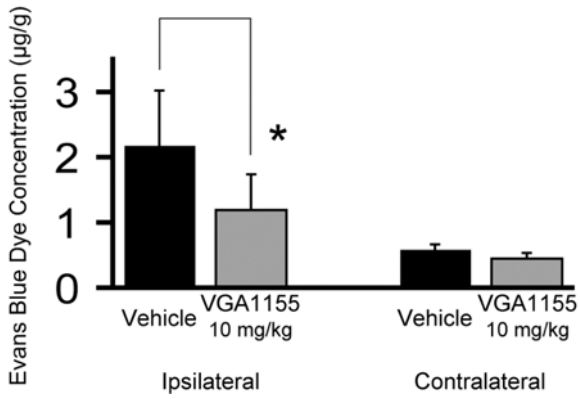


Figure 2. Results of Evans blue extravasation measured 6 h after MCAO. VGA1155 treatment significantly reduced the Evans blue extravasation. VGA1155 (10 mg / kg) was given at 30 minutes before MCA occlusion. Values are expressed as means ± S.D. (n = 5, in each group). * $P < 0.05$.

Infarct volumes

In the control group, the infarct volume was 36.7 ± 6.2 % (n = 5, Figure 3.), measured at 7 days after the reperfusion. Administration of VGA1155 at 30 minutes before cerebral ischemia significantly reduced the infarct volume (22.5 ± 9.0 % (n = 5), $P < 0.05$, Figure 3.) compared with the control group.

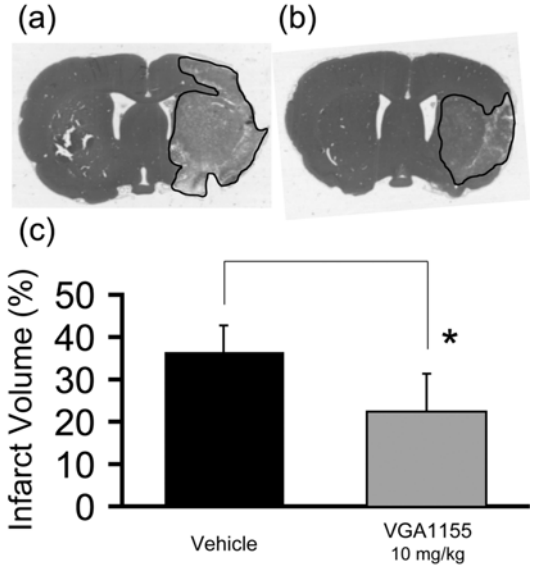


Figure 3. (a), (b) Representative hematoxylin and eosin-stained brain section showing infarct lesion one week after transient focal cerebral ischemia in Vehicle (a) and VGA1155-treatment (10 mg / kg) (b) group. (c) Results of %-infarct volume 7 days after MCA occlusion. Preischemic treatment with VGA1155 significantly reduced the infarct volume. Vehicle and VGA1155 were given (i.p.) 0.5 h before MCA occlusion. Values are expressed as means ± S.D. (n = 5, in each group). * $P < 0.05$.

Neurological score

Changes of neurological scores in the two groups are shown in Figure 4. At 24 hours after the ischemic injury, there is no difference in neurological scores between vehicle control rats and rats treated with VGA1155 (2.6 ± 0.5 (n = 5), 3.0 ± 0.7 (n = 5), NS, Figure 4.). Neurological score of VGA1155-treated animals at 7 days after cerebral ischemia was significantly improved compared with the score of the vehicle-treated animals (0.2 ± 0.4 (n = 5), 2.0 ± 1.0 (n = 5), $P < 0.01$, Figure 4.).

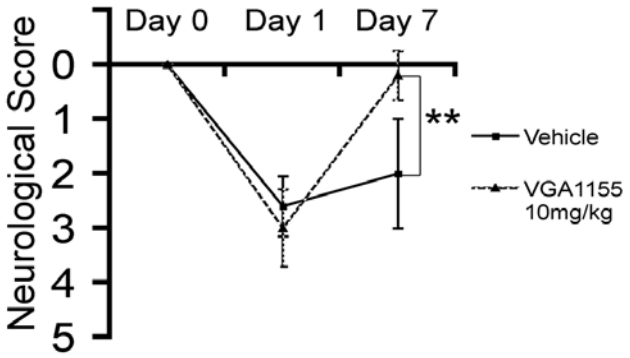


Figure 4. Effect of VGA1155 on neurological score after ischemic injury. Although VGA1155 did not affect the neurological scores 24 h after MCA occlusion, VGA1155 significantly improved the neurological scores 7 days after MCA occlusion. Values are expressed as means ± S.D. (n = 5, in each group). ** $P < 0.01$.

DISCUSSION

The present study has demonstrated that VGA1155 at 30 minutes before focal cerebral ischemia significantly reduced the vascular permeability and brain edema at 24 hours after the ischemia. This effect was maximized at a dose of 10 mg / kg. Furthermore, the administration of VGA1155 reduced infarct volume and improved neurological deficits at 7 days after the cerebral ischemia.

Previous studies have demonstrated upregulation of VEGF expression following the ischemic insult in the rodent. It has been reported that rapid induction of VEGF gene expression after transient middle cerebral artery occlusion in rats [10]. It has also been reported that VEGF mRNA to be upregulated as early as 3 hours after ischemia with a peak between 24 and 48 hours [16]. VEGF-induced vascular permeability can aggravate brain edema. Therefore, it was suggested that VEGF antagonism in acute phase of cerebral ischemia plays a protective role against brain edema. Another aspect of VEGF than increasing vascular permeability is vasculogenesis. VEGF would play a great role to establish neovascularization and functional recovery after ischemic injury. VEGF antagonism may inhibit this process and exacerbate ischemic injury. Some previous studies reported that exogeneous VEGF has neuroprotective effect in ischemic brain. Harrigan et al. reported that intraventricular infusion of VEGF for 7 days prior to MCAO reduces infarct volume and brain edema [9]. Sun et al. reported that administration of VEGF by the intracerebroventricular route from one day after induction of ischemia reduces infarct volume and improves neurological outcome at 3-28 days after transient focal ischemia [23].

Zhang et al. reported that intravenous administration of VEGF 48 hours after MCAO to rats improved neurological function at 7-28 days, however, administration of VEGF 1 hour after MCAO aggravated [28]. These suggest that both of VEGF and VEGF antagonism have neuroprotective effects if administered under the proper conditions. We chose 30 minutes prior to MCAO as the timing for VGA1155 administration since a pilot study (data not shown) at the manufacturing company showed that the half-life period of VGA1155 in rat plasma was 21 hours after intraperitoneally injection. Theoretically acute cerebral edema after ischemia can be treated within this time period. The study to demonstrate therapeutic time window is of our future interest.

Various agents have previously been reported to show VEGF antagonism, including anti-VEGF antibody [11, 12], anti KDR/Flk-1 antibody [20], 2'-fluoropyrimidine RNA-based aptamers [21], various peptides [2, 5], porphyrin analogues [1], mFlt1(1-3)-IgG [26] and soluble Flt-1 [14]. Compared with these agents, VGA1155, with its low molecular weight, shows certain advantages, including low cost of synthesis, low antigenecity and in vivo stability. VGA1155 is synthesized to inhibit binding between VEGF and its two receptors, Flt-1 and KDR/Flk-1. Furthermore, VGA1155 does not inhibit the binding of other ligands to their receptors, such as EGF, PDGF or other cytokines, which indicates the highly specific nature of the inhibitory effects of VGA1155 on VEGF binding to both receptors [25]. Many of other VEGF antagonists are tyrosin kinase inhibitors and are required to enter the cytosol to exert their effects. In contrast, VGA1155 inhibits the binding of VEGF to the receptor on the cell surface and may be able to exert its effect without entering the target cells. This suggests that VGA1155 shows a lower toxicity than other VEGF antagonists. Two previous studies showed that VEGF blockade has beneficial effects in a rodent model of cerebral ischemia. One required multiple courses of a soluble VEGF receptor treatment [26]. The other required the delivery of soluble Flt-1 gene into the cerebral ventricle [14]. In the present study, intraperitoneal single administration of VGA1155 significantly reduced brain edema and infarct volume. We believe that VGA1155 is a suitable pharmacological antagonist as a therapeutic tool.

We also showed reduction of the impaired area and neurological score. The rescue of brain damage was expectedly large and there might be some other beneficial effect of VGA1155 than the reduction of acute brain edema. Shimaura et al. has shown inhibition of integrin β3 improves outcomes and decreases inflammatory cells in the penumbra in rat focal cerebral ischemia model [22]. VEGF is also known to have pro-inflammatory effects and enhance macrophage activity [27]. Early antagonism of VEGF after cerebral ischemia may inhibit infiltration of inflammatory materials causing neuronal damage.

VGAs were originally developed to suppress angiogenesis in cancer and therefore there may be the potential side effects in late phase. Microvessel development and increase in blood flow in ischemic brain may be reduced. Obtained results showed rather reduction of infarct volume and improve of neurological score at one week, although there might be some adverse effect owing to possible anti-vasculogenesis effect. For clinical trial use of this drug, safety should be confirmed using larger animal models which should be observed at least for months after administration.

In conclusion, we have shown that pre-ischemic blockade of VEGF receptors by VGA1155, a novel low molecular weight antagonist, reduces brain edema by decreasing BBB permeability in a rat model of transient focal cerebral ischemia. Furthermore, the treatment reduces infarct volumes and improves neurological outcome a week after the ischemia.

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