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Effect of VEGF Receptor Antagonist (VGA1155) on Brain Edema in the Rat Cold Injury Model

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Vascular endothelial growth factor (VEGF) is a major mediator of angiogenesis and a strong vascular permeability factor. Blockade of VEGF may have a potential to treat brain edema after brain injury. In the rat cold injury model, the VEGF receptor antagonist VGA1155 significantly reduced the brain water content and the maximum effect was obtained when given at 30 minutes after injury. This effect was in dose-dependent manner and the dose of 25 mg/kg showed the maximum effect. With this dose, VGA1155 also significantly reduced vascular permeability. Histological evaluation of brain lesion showed no significant reduction of damaged area 1 week after injury by VGA1155. The data suggest that VGA1155 may have antiedematous effect in acute phase after cold injury.

Brain edema has been theoretically classified into two types depending on the mechanisms; cytotoxic edema and vasogenic edema. Vasogenic edema is caused by disruption of the blood-brain barrier (BBB). It is associated with various primary brain damage, such as cerebral ischemia, traumatic brain injury, brain tumor and encephalitis. The prolonged brain edema exacerbates brain damage in the area surrounding the primary lesion. Conventional treatments are performed clinically with drugs including dehydration to reduce the brain edema but the effect is incomplete. Development of more effective medical treatment has been expected.

Vascular endothelial growth factor (VEGF) is a well-known major inducer of angiogenesis on the endothelial cells. VEGF is also known to increase vascular permeability in a short time period [1,2,4]. The latter effect may increase brain edema and deteriorate the brain damage in cerebral ischemia or traumatic brain injury [20]. Therefore, VEGF blockade may reduce brain edema after brain insult and has a potential to become a new therapeutic approach to manage brain edema. VEGF exerts its effect mainly via the two high-affinity tyrosine kinase receptors, Flt-1 (VGFR1) and KDR/Flk-1 (VGFR2). Recently, several experimental studies have revealed antiedematous effect by suppressing VEGF pathway [9, 20]. In the present study, we evaluated effects of VGA1155 (5-[N-Methyl-N-(4-octadecyloxy-phenyl)acetyl]amino- 2-methylthiobenzoic acid), a newly developed binding antagonist of VEGF, on brain edema in the rat cold injury model. VGA1155 is synthesized to inhibit binding between VEGF and its two receptors, Flt-1 and KDR/Flk-1 [17,18]. The cold injury model, established by Klatzo et al. [8], has been one of the consistent models for the study of vasogenic edema [2,6].

Brain edema and vascular permeability were assessed by measuring the water content and Evans blue. We found that VGA1155 reduced brain edema and vascular permeability in

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the rat cold injury model. Our results demonstrate that the VEGF receptor antagonist has a potent therapeutic effect in the cold injury model.

MATERIALS AND METHODS

Experimental animals

In total, eighty rats were used in this study. All procedures for these experiments were approved by the Committee on Animal Experimentation, Kobe University Graduate School of Medicine and performed in strict accordance with the related guidelines. Male Fischer 344 rats weighing 160–230 g (CLEA Japan, Inc.; Osaka, Japan) were used for this study. The rats were housed in a controlled environment (alternating 12 h light/dark cycle, 22 ± 2 °C, 55 ± 5% relative humidity) and allowed free access to food and tap water throughout the experiments. The rats underwent surgery in random order and evaluation of results was made by investigators blinded to the experimental groups.

Rats were anesthetized by the intraperitoneal injection of sodium pentobarbital (50 mg/kg) and positioned in a stereotactic frame. After a midline incision of the scalp was made, the skull was exposed. Cold injury was made by a liquid nitrogen-cooled copper tube probe with 4 mm in diameter, contacting on the skull, at 2.5 mm lateral to the midline and 2mm posterior to the coronal suture, for 30 seconds. The rectal temperature of all animals was maintained at 36.5 °C to 37.5 °C using a heating pad. Sham-operated rats underwent the same procedure except for the induction of the cold injury and used as the control. Rats were sacrificed at a designated time point under deep anesthesia by the intraperitoneal injection of sodium pentobarbital (100 mg/kg). To verify the physiological parameters, rats in four groups (control rats treated with vehicle, control rats treated with VGA1155, cold injury rats treated with vehicle and cold injury rats treated with VGA1155, n = 4 in each group) were studied for the systemic arterial blood pressure, arterial blood gases, plasma electrolytes and osmolality measurements by cannulating the femoral artery.

VGA1155

VGA1155 was synthesized at the Research Center, Taisho Pharmaceutical Co., Ltd., Saitama, Japan. For this experiment, VGA1155 was dissolved in isotonic phosphate buffer (pH 9.0) and administered by intraperitoneal injection. Rats were randomly allocated into injured rats treated with phosphate buffer (pH 9.0) or injured rats treated with VGA1155. Phosphate buffer was injected intraperitoneally 0.5 h after cold injury in the vehicle group. In the dose-effect study, rats receiving cold injury were treated intraperitoneally with VGA1155 25 mg/kg at 0.5 h after injury. In the time-window study, rats receiving cold injury were treated intraperitoneally with VGA1155 0.5 h before, 0.5 h or 4 h after cold injury. In the treatment group, VGA1155 was given either 0.5 h before cold injury or 30 minutes or 4 h after cold injury. Four sham operated rats received intraperitoneal injections of VGA1155 at a dose of 50 mg/kg in order to examine the systemic effects of the drug.

VEGF immunoassay

The expression of VEGF after cold injury was measured by immunoassay (n = 20). Rats were sacrificed at different time points (0.5, 12, 24 and 48 h) after the cold injury. Tissue samples were obtained from the periphery of the cold-injured lesion (n = 4 in each time point). For the controls, samples were also obtained from the corresponding areas in the animals without injury (n = 4).

The tissues were homogenized in the RIPA buffer (50 mM Tris-HCl, pH 7.4, 1 % NP-40, 0.5 % Na-deoxycholate, 150 mM NaCl, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM Na_3VO_4, 1 mM NaF). The homogenates were centrifuged at 15,000 rpm at 4 °C for 20 minutes and the supernatants were collected. Protein concentrations of

the supernatants were determined by the Bradford method using BSA as the standard. The supernatants were diluted to adjust the protein concentration. VEGF were then quantitated using a commercially available VEGF ELISA kit (Quantikine M; R&D systems) according to the manufacturer's instruction.

Measurement of brain water content

Brain edema was evaluated by measuring the water content (n = 40). Rats were sacrificed by decapitation under deep anesthesia at 24 h after the cold injury. The brains were removed rapidly and divided into hemispheres. Each hemisphere was weighed to obtain the wet weight, and then dried at 110 °C for 24 h. The water content in the hemisphere was calculated as follows: water content (%) = (wet weight – dry weight)/wet weight × 100. For dose-dependent study, VGA1155 was administrated at 30 minutes after injury in various doses (5, 25, 50 mg/kg and vehicle, n = 5 for each). The control rats with sham operation were also treated by either vehicle or 50 mg/kg of VGA1155 (n = 5 for each).

Blood-brain barrier permeability to Evans blue

Vascular permeability was assessed with Evans blue dye (n=20). The rats were administered intravenously with 2 ml/kg of 2% Evans blue solution immediately after the operation. Twenty-four hours after the injury, the rats were deeply anesthetized and transcardialy perfused with saline at the pressure of 90 mmHg pressure until colorless perfusion fluid was obtained from the right atrium. After decapitation, brains were removed quickly and each cerebral hemisphere was separated. The tissue was weighed, homogenized in 1.5 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 measured by a luminescence spectrophotometer (excitation at 620 nm and emission at 680 nm). 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 weight (g).

Measurement was performed in sham-operated rats with vehicle (n = 5), in cold injury rats with vehicle (n = 5), and in cold injury rats with VGA1155 treatment at the dose either 25 mg/kg (n = 5) or 50 mg/kg (n = 5). VGA1155 or vehicle was applied 30 minutes after cold injury.

Assessment of lesion volume

One week after the surgery, assessment of cortical lesion volume was performed in cold injury rats with vehicle (n = 5), and in cold injury rats with VGA1155 treatment at the dose either 25 mg/kg (n = 5). Rats were sacrificed by over-dose anesthesia and transcardially perfused with saline and then 4% paraformaldehyde in 0.1 mol/L sodium phosphate, pH7.4. Brains were carefully removed, fixed in 4% paraformaldehyde solution and dehydrated in phosphate-buffered 30% sucrose. Paraffin-embedded brains were then sectioned in 10 μm thickness at 0.25 mm intervals from 1 mm anterior to 3.5 mm posterior to the bregma and stained with 0.1 % cresyl violet. Lesion areas were measured using Image Pro Plus Ver. 5.0 (Media Cybernetics, Inc) and total lesion volume were calculated as the sum of the product of the lesion areas times the thickness of the sections.

Statistical analysis

All values are expressed as means ± standard deviation (SD). Physiological parameters and VEGF immunoassay were analyzed by one-way ANOVA followed by post hoc comparison with the use of the Bonferroni/Dunn test. Water content, Evans blue extravasation and lesion volume were analyzed by using the unpaired Student's *t* test. Statistical significance was accepted at $P < 0.05$.

RESULTS

Physiological parameters

There were no significant differences of the arterial blood pressure, arterial blood gas, blood pH, blood glucose concentration, plasma electrolytes or osmolality among the experimental groups at each time point (Table 1). Body weight loss was significantly greater in the cold injury groups than in the sham-operated group. VGA1155 did not affect the loss of body weight.

Table 1. Physiological Parameters [a]

	Blood pressure (mm Hg)	PCO_2 (mm Hg)	PO_2 (mm Hg)	pH	Glu (mg/dl)	Na^+ (mEq/l)	K^+ (mEq/l)	Osmolality (mOsm/l)	Weight loss (g)
Sham operated									
Preop	101.2±8.7	40.6±3.9	77.0±7.8	7.44±0.01	162.5±23.8	139.0±1.4	3.8±0.4	301.8±2.5	
Postop	100.6±10.3	40.4±2.5	80.0±4.7	7.43±0.03	157.3±37.0	140.8±2.6	3.6±0.3	304.4±4.1	
24h	97.4±7.7	40.6±1.4	81±5.4	7.43±0.01	141.3±6.9	140.0±0.8	3.2±0.3	300.4±1.1	6.3±3.4
VGA1155-treated (50mg/kg) in normal rat									
Preop	98.5±3.3	40.8±5.7	70.3±5.9	7.42±0.04	168.8±13.3	140.8±1.5	3.9±0.6	305.0±1.3	
Postop									8.0±6.2
24h	98.8±15.4	37.8±3.7	75.3±7.0	7.45±0.02	142.5±19.1	136.8±6.8	3.8±1.1	300.7±6.0	
Vehicle									
Preop	100.7±4.4	40.5±5.5	75.3±5.9	7.44±0.03	148±11.9	141.3±1.0	3.7±0.3	302.6±1.5	
Postop	99.5±2.8	39.4±1.7	84.3±6.3	7.44±0.03	148.5±12.3	140.5±3.7	3.4±0.6	303.6±3.8	
24h	92.2±6.5	37.7±1.8	71.0±6.3	7.46±0.02	148.3±18.3	137.8±9.0	4.0±1.5	296.0±14.5	12.3±2.2 [c]
VGA1155-treated (50mg/kg) [b] at 0.5 h after cold injury									
Preop	102.4±8.1	39.5±6.8	69.5±3.7	7.41±0.02	148.8±15.4	141.8±3.5	3.6±0.3	307.9±5.1	
Postop	103.2±10.8	43.3±5.0	76.0±1.8	7.41±0.02	151.8±10.6	142.3±1.3	3.3±0.2	306.1±2.9	
24h	95.7±5.7	42.8±3.6	70.0±5.1	7.42±0.05	123.0±11.5	142.3±4.1	3.2±0.4	302.1±4.5	12.4±2.5 [c]

[a] Values are expressed as means ± S.D. (n = 4, in each group). There were no significantly differences in arterial blood gases, blood glucose concentration, osmolality or electrolytes of the blood plasma among the groups.
[b] VGA1155 was administered at 0.5 hour after the cold injury.
[c] $P < 0.01$ versus sham-operated group.

Serial change in VEGF level after the cold injury

The immunodetectable VEGF protein concentration was evaluated between 0.5 h and 48 h after cold injury. The cortical VEGF level was 1652 ± 212 pg/g total protein in sham-operated animals. VEGF in the periphery of the cold injury was significantly increased to 2866 ± 933 pg/g at 0.5 h and 2772 ± 346 pg/g at 48 h (Fig. 1).

Brain water content

VGA1155 did not affect the brain water content in sham-operated animals (Fig. 2a). The water content of the ipsilateral hemisphere was significantly increased after cold injury to 80.6 ± 0.3 %, compared to that of the sham-operated animal (78.6 ± 0.1 %, $P < 0.01$, Fig. 2a). In dose-effect studies, VGA1155 doses of 25 and 50 mg/kg given 0.5 h after cold injury significantly attenuated the increase of water content (79.3 ± 0.4 %, 79.4 ± 0.6 %, $P < 0.01$). In time-window studies, the water content was significantly reduced when VGA1155 was given 0.5 h after cold injury (Fig. 2b). Delayed treatment at 4 h after cold injury, however, did not attenuate the edema induced by cold injury.

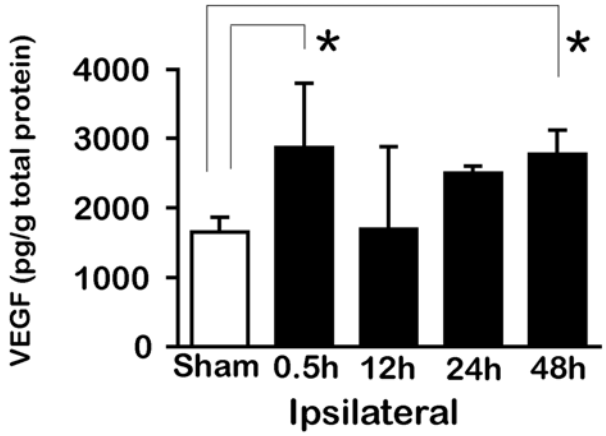


Fig. 1. The expression of VEGF protein in the sham-operated and the cold-injured rats. Cortical homogenates from the rats at different times after the cold injury were prepared. VEGF in the homogenates was assessed using VEGF ELISA kit. Values are expressed as means ± SD (n=4, in each group). *, $P < 0.05$, significantly different from the sham-operated group (ANOVA with Bonferroni/Dunn test).

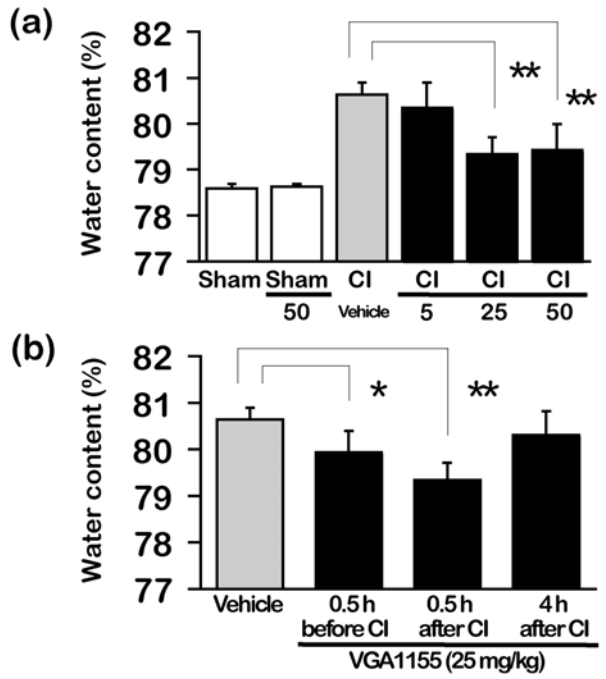


Fig. 2. Water content was measured 24 hours after the cold injury. Values are expressed as means ± SD (n = 5, in each group).
(a) In dose-effect studies, VGA1155 doses of 25 and 50 mg/kg given 0.5 h after cold injury significantly attenuated the increase of water content (** *P < 0.01*).
(b) In time-window studies, the water content was significantly reduced when VGA1155 was given 0.5 h after cold injury (** *P < 0.01*) or 0.5 h before injury (* *P < 0.05*).

Blood-brain barrier permeability to Evans blue

The blood-brain barrier permeability to Evans blue was evaluated 24h after cold injury in the control (Fig. 3a, 3b). The degree of blood-brain barrier disruption, as indicated by the extravasation of Evans blue, was significantly greater in the ipsilateral hemisphere after the cold injury (3.6 ± 0.7 μg/g of wet tissue) compared to that of the sham-operated group (1.2 ± 0.1 μg/g of wet tissue, $P < 0.01$). The Evans blue permeability did not change in the hemisphere contralateral to the cold injury. VGA1155 treatment significantly reduced the degree of BBB disruption. It was 2.0 ± 0.5 μg/g of wet tissue for 25 mg/kg of VGA1155 ($P < 0.01$) and 2.4 ± 0.4 μg/g of tissue for 50 mg/kg of VGA1155 ($P < 0.05$) (Fig. 3b).

Assessment of lesion volume

Although mean cortical lesion volume at 7 days after cold injury was reduced in VGA1155-treatment group (2.6 ± 1.5 mm^3) compared with vehicle (3.1 ± 1.8 mm^3), this difference did not reach statistical significance ($P = 0.66$, Fig. 3c, 3d).

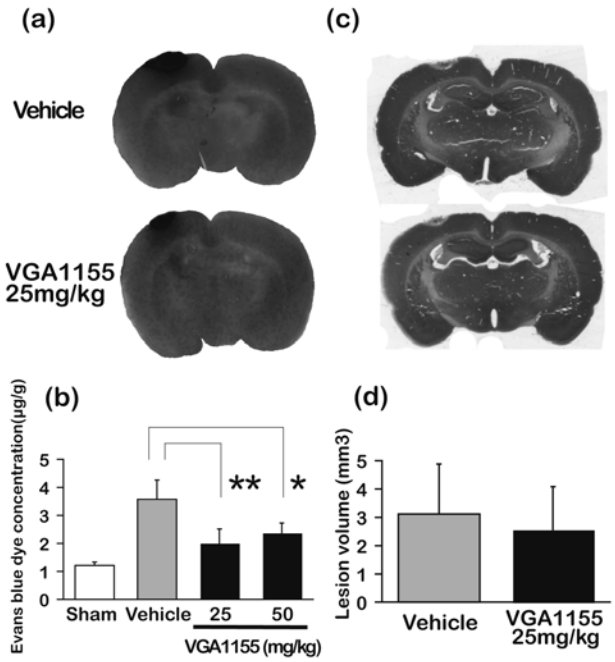


Fig. 3. (a) Representative brain sections showing extravasation of Evans blue 24 h after cold injury in Vehicle and VGA1155-treatment (25 mg/kg) group.
(b) VGA1155 treatment significantly reduced the degree of Evans blue permeability (25 mg/kg ** $P < 0.01$, 50 mg/kg * $P < 0.05$).
(c) Representative Nissl-stained brain sections showing cortical damage one week after cold injury in Vehicle and VGA1155-treatment (25 mg/kg) group.
(d) Cortical lesion volumes assessed one week after cold injury. There was no significant difference between vehicle and VGA1155 (25 mg/kg) treatment group.

DISCUSSION

The results of this study show that VGA1155, a novel binding antagonist of VEGF to its receptor, reduced the vascular permeability and attenuated the brain edema in a

dose-dependent manner in the rat cold injury model. The present results suggest that early VEGF antagonism may be useful in the treatment of vasogenic edema following brain injury.

VEGF is well known to be the major inducer of angiogenesis. However, it is also a potent mediator of vascular permeability [1, 3, 4]. Many reports have demonstrated that VEGF expression associated with edema formation in brain tumors [10, 11], brain injury [12, 13, 14] and various types of cerebral ischemia [5, 15, 16, 20]. Van Bruggen et al. [19] reported that the antagonist of VEGF with a fusion protein mFlt(1-3)-IgG, which sequesters murine VEGF, reduced ischemia/reperfusion-related brain. Kimura et al. [7] demonstrated that a VEGF antagonist attenuated the vascular permeability and reduced cerebral venous infarction in a rat two-vein occlusion model. Lenzlinger et al. [9] reported recently that the inhibition of VEGF receptor signaling by BSF476921 attenuated regional brain edema following traumatic brain injury in rats. Thus VEGF receptor antagonists promise to be new therapeutic tools of brain edema. Therefore we hypothesized that inhibition of the VEGF effect may be beneficial in the reduction of the vasogenic edema induced by cold injury.

VGA1155 is synthesized to inhibit binding between VEGF and its two receptors, Flt-1 and KDR/Flk-1. In the previous study, Ueda et al. [17] showed the anti-angiogenic effect of VGA1155 both *in vitro* and *in vivo*. Additionally, Ueda et al. [18] reported that VGA1155 can reduce the vascular permeability induced by the intradermal injection of VEGF using the Miles assay. In the present study, we consider that the anti-angiogenic effect of VGA1155 is not related to the detrimental effects since VGA1155 was administered in the extremely acute phase after the injury. 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 [18]. In our present study, VGA1155 did not affect any physiological parameter. VGA1155 seems to show the antiedematous effect in the rat cold injury model without any serious side effect.

Systemic administration of VGA1155 attenuated brain edema and vascular permeability induced by cold injury in acute phase, however, VGA1155 did not reduce the lesion volume at 7 days after cold injury. One possible explanation might be the fact that brain edema induced by cold injury is reversible in most and the lasting lesion is relatively small. Previous studies have shown that BBB breakdown after cold injury occurs in two phases and the latter one is mediated by several vasoactive agents including VEGF. However, the present study demonstrated that VEGF is upregulated 30 min after cold injury in the ipsilateral hemisphere. Delayed administration at 4 h after cold injury failed to reduce the edema. Thus, antagonism of the VEGF receptor is necessary before the induction of VEGF protein to reduce brain edema.

Our results showed that the VEGF receptor antagonist VGA1155 is effective in the treatment of cold-induced vasogenic brain edema. VEGF receptor antagonists may herald the dawn of a new age in the therapy of vasogenic edema induced by various kinds of brain injury.

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