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Full paper

Effects of kallidinogenase on retinal edema and size of non-perfused areas in mice with retinal vein occlusion

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A B S T R A C T

Kallidinogenase has been used to treat retinal vein occlusion (RVO) in patients, although there are no evidences on the effects of kallidinogenase on the retinal edema and the non-perfused areas in eyes with a RVO. We have established a murine RVO model with retinal edema and non-perfused areas. The purpose of this study was to evaluate the effects of kallidinogenase on the retinal edema and size of the non-perfused areas in the mouse RVO model. We evaluated the thickness of the retinal layers and size of the non-perfused areas, and the blood flow by laser speckle flowgraphy in RVO model. The effects of an intravenous injection of kallidinogenase on the retinal edema and size of the non-perfused areas were determined. In addition, the expressions of phosphorylated protein kinase B (Akt) and endothelial nitric oxide synthase (eNOS) were measured by Western blotting. Our results showed that kallidinogenase reduced the degree of retinal edema and size of the non-perfused areas by an increase in the blood flow in RVO model. Kallidinogenase also increased the levels of phosphorylated Akt and eNOS. These findings indicate that kallidinogenase acted through Akt/eNOS-dependent phosphorylation. Thus, kallidinogenase should be considered as a possible therapeutic agent for RVO patients.

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1. Introduction

A retinal vein occlusion (RVO) is a relatively common retinal vascular disorder that can lead to retinal hemorrhages and macular edema which, in turn, can cause vision impairments.[1,2] Anti-vascular endothelial growth factor (anti-VEGF) compounds are the only approved treatment for eyes with a RVO. However, there are problems with anti-VEGF compounds including contradictory findings on its effect on the signs and symptoms of RVO. For example, some studies have reported that anti-VEGF compounds reduced the size of the non-perfused area,[3] however other reports suggested that anti-VEGF compounds increased the size of the non-perfused areas.[4] Moreover, some patients did not respond to the anti-VEGF compounds, and others had repeated recurrences of edema and non-perfused areas after the anti-VEGF treatment.[5]

A second reason against anti-VEGF therapy is that repeated injections of anti-VEGF compounds are needed, and this can be associated with endophthalmitis and economic burdens.[6] Hence, anti-VEGF therapy often leads to low compliance.

Because of these problems, it is important to search for alternative therapies for RVO patients. However, it is difficult to perform clinical research to determine the effects and mechanisms for the treatments on RVO patients due to complexities of the patient's conditions. To overcome this limitation, we have developed a murine RVO model that has many of the manifestations of RVO in humans, e.g., cystoid edema, retinal hemorrhages, and retinal non-perfused areas.[7] This model can then be used to examine the effects of new agents on the damages induced by RVO.

One of the agents is kallidinogenase which is derived from porcine pancreas, and it belongs to a group of serine proteinases that release kinin, a potent vasodilator peptide, from plasma kininogens.[8] The administration of kallidinogenase has been shown to improve several eye diseases.[9,10] Endogenous kallidinogenase is associated with the formation of bradykinin, which can increase the diameter of blood vessels,[11] and the kallikrein-kinin system is associated with various vascular disorders.[12,13] The administration of kallidinogenase has been shown to improve the choroidal and

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retinal circulation in a retinal hypertensive rabbit model and after ischemic injuries induced by endothelin-1.[14,15] It has also been reported that kallidinogenase can suppress the development of edema by normalizing the retinal vasopermeability in streptozotocin-induced diabetic rats.[16] The results of that study showed that treatment with kallidinogenase can increase the blood flow which can then prevent retinal angiogenesis. Furthermore, we have reported that intravenous kallidinogenase led to a significant decrease of retinal neovascularization, and it may have an anti-angiogenic effects through the selective cleavage of $VEGF_{165}$.[17]

Kallidinogenase has been used to treat RVO patients. However, the effects and the mechanisms of kallidinogenase against the RVO are poorly understood. Therefore, the aim of this study was to determine the effects and mechanisms of action of kallidinogenase on the degree of retinal edema and size of non-perfused areas in mice with RVO.

2. Material and methods

2.1. Animals

Eight-weeks-old male ddY mice were purchased from Japan SLC (Hamamatsu, Japan). The animals were housed at 23 °C ± 3 °C, under a 12 h light/dark cycle (lights on from 8:00 to 20:00 h). All experiments were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and were approved and monitored by the Institutional Animal Care and Use Committee of Gifu of Pharmaceutical University.

2.2. Retinal vein occlusion (RVO) model

The RVO model was produced as described in detail.[7] Briefly, mice were anesthetized by an intramuscular injection of a mixture of ketamine (120 mg/kg; Daiich-Sankyo, Tokyo, Japan) and xylazine (6 mg/kg; Bayer, Health care Osaka, Japan). The pupils were dilated with 1% tropicamide and 2.5% phenylephrine (Santen Pharmaceuticals Co., Ltd.). After confirming that the anesthesia was adequate, rose bengal (8 mg/mL; Wako, Osaka, Japan) was injected into a tail vein. A few minutes later, hydroxyl ethyl cellulose (Senju Pharmaceutical Co. Ltd., Osaka, Japan) was applied topically to the cornea to prevent desiccation and to keep the surface smooth. Then, 10 to 15 laser spots (three veins/animal) were applied to a branch vein (3 disc diameters from the optic nerve centres) of the right eye of each animal. The image-guided laser system (532 nm) attached to the Micron IV Retinal Imaging Microscope (Phoenix Research Laboratories, Inc., Pleasanton, CA, USA) was used at 50 mW power, 5000 ms duration, and 50 μm size.

2.3. Drug administration

Kallidinogenase was obtained from Sanwa Kagaku Kenkyusyo Co. Ltd. (Aichi, Japan). After confirming that the anesthesia was adequate, kallidinogenase was injected into a tail vein. The kallidinogenase-treated mice were divided into two groups. Mice in the first group were given 10 μg/kg of kallidinogenase twice a day for 2 days before the laser irradiation, and then immediately and 12 h after the laser exposure (pre/post RVO + kallidinogenase). Mice in the second group were given 10 μg/kg kallidinogenase immediately and 12 h after the laser photocoagulation (post RVO + kallidinogenase).

2.4. Optical coherence tomography imaging

The retinal thickness was measured by optical coherence tomography (OCT) as reported in detail.[7] Images were captured from 20 positions for the right eye by the StreamPix 6 and Micron OCT commercial software (Phoenix Research Labs). The retinal thickness was measured at 20 recorded positions by the Insight software and the average of all positions represented the overall retinal thickness.

2.5. Histological analysis

Histological analysis was performed on representative mice from the two groups. To accomplish this, the mice were anesthetized, and the eyes were enucleated and placed in a fixative solution containing 4% paraformaldehyde (PFA) for at least 48 h at 4 °C. Six paraffin-embedded sections (5 μm) were cut through the optic disc of each eye, prepared in the standard manner, and stained with hematoxylin and eosin. The damage induced by the retinal vein occlusion was then evaluated in six randomly selected areas from each eye for the morphometric analysis. Images were photographed with a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan). The thickness of the inner nuclear layer (INL) and the outer nuclear layer (ONL) were measured on the photographs every 240 μm from the optic disc toward the periphery with ImageJ (National Institutes of Health, Bethesda, MD, USA). The data from three sections selected randomly from the six sections were averaged for each eye.

2.6. Imaging of retinal non-perfused areas

Mice were injected with kallidinogenase intravenously immediately or 7 days after the laser occlusion, and the retinas were collected on days 1 and 7 after the injection of kallidinogenase. The mice were injected with 0.5 mL of 20 mg/mL fluorescein conjugated dextran (Sigma–Aldrich Corp., St. Louis, MO, USA) dissolved in PBS into the tail veins before the sampling. Eyes were enucleated and fixed for 7 h in 4% paraformaldehyde, and retinal flat-mounts were prepared. Images of the flat-mounted retinas were taken with Metamorph (Universal Imaging Corp., Downingtown, PA, USA). The ImageJ processing software was used to determine the size of the retinal non-perfused areas.

2.7. Blood flow measurements determined by laser speckle flowgraphy

The blood flow on the optic nerve head (ONH) was measured by laser speckle flowgraphy (LSFG; Softcare Co., Ltd., Fukuoka, Japan) as reported in detail.[7] The blood flow was expressed as the mean blur rate (MBR) which is an index of the relative blood flow velocity.[18] The MBR images were acquired continuously at a rate of 30 frames per second over a time period of approximately 4 s. The measured fundus area was approximately 3.8 × 3 mm (width × height) with an estimated tissue penetration of 0.5–1 mm. After the image acquisition, the vessel and tissue areas on the ONH area were automatically detected by the LSFG Analyzer software (version 3.1.14.0; Software Co., Ltd.) using the so-called vessel extraction function. The MBRs in the different regions of the ONH were calculated using the software which was divided into three parameters; the MBR of the total area (MA) was calculated as the average MBR over the entire ONH, the MBR of the vessel region (MV) was the average of the vessel region, and the MBR of the tissue region (MT) was calculated as the average MBR of the total ONH area minus the vessel region.

2.8. Western blot analysis

Western blot analysis was performed as reported.[19] The primary antibodies were; rabbit anti-phospho-protein kinase B (1:1000; p-Akt; Cell Signaling, Danvers MA, USA), rabbit anti-total-Akt

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