Tissue Kallikrein Inhibits Retinal Neovascularization via the Cleavage of Vascular Endothelial Growth Factor-165

Shinsuke Nakamura, Nobutaka Morimoto, Kazuhiro Tsuruma, Hiroshi Izuta, Yoshika Yasuda, Noriaki Kato, Tsunehiko Ikeda, Masamitsu Shimazawa, Hideaki Hara

Objective—Tissue kallikrein, a widely used vasodilator for the treatment of hypertension and peripheral circulatory disorders, acts by releasing kinin, a potent vasodilator peptide. To identify the role of tissue kallikrein in retinal neovascularization, we investigated the antiangiogenic effect by using an in vitro and in vivo angiogenesis model.

Methods and Results—Tissue kallikrein in vitreous fluid was markedly elevated in proliferative diabetic retinopathy patients compared with that in control patients with macular hole and epiretinal membrane. Tissue kallikrein inhibited vascular endothelial growth factor-165 (VEGF165)-induced tube formation, proliferation, and migration in vitro angiogenesis model via suppression of the VEGF165-induced phosphorylation of VEGF receptor-2. Furthermore, tissue kallikrein cleavage of VEGF165 was on the C-terminal side, which was analyzed by Western blotting and mass spectrometry. When administered subcutaneously, tissue kallikrein reduced the pathological vascular changes in retinal neovascularization induced in neonatal mice by returning the retina to normoxia after exposure to hyperoxia.

Conclusion—These findings indicate that tissue kallikrein is partly involved in pathogenesis of proliferative diabetic retinopathy and may be a promising therapeutic agent that could cleave VEGF165 itself when administered by a peripheral route. (Arterioscler Thromb Vasc Biol. 2011;31:1041-1048.)

Key Words: angiogenesis ■ pharmacology ■ vascular biology

Irreversible vision loss and blindness has been increasing because of abnormal retinal neovascularization. Retinal neovascular diseases, including diabetic retinopathy, age-related macular degeneration, and retinopathy of prematurity, are the major causes of blindness worldwide.1–3 Especially in the advanced countries, lifestyle changes and superaging have led to a rapid rise in the number of cases of diabetic retinopathy and age-related macular degeneration.

An extensive proliferation of new blood vessels in the retina is induced by a specific cytokine, vascular endothelial growth factor (VEGF).4,5 Recently, intravitreal injection of an anti-VEGF antibody, a VEGF aptamer, and a VEGF Fab fragment has been used in the treatment of age-related macular degeneration, proliferative diabetic retinopathy (PDR), central retinal vein occlusion, and neovascular glaucoma.6–9 However, repeated injections are associated with potential risks of vitreous hemorrhages, retinal detachment, and decrease in compliance.10,11 Therefore, noninvasive delivery systems, such as peripheral administration, are required.

The kallikrein-kinin system is present in various vascular territories in both humans and mice.12–14 Tissue kallikrein endogenously forms kinins, such as bradykinin, which can participate in the regulation of vascular tone.15–17 Tissue kallikrein has been demonstrated to improve choroidal and retinal circulatory disorders in the renal hypertensive model18 and in ischemic injury induced by endothelin-1,19 one of the most potent endogenous vasoconstrictors.20 We have recently reported that tissue kallikrein administered intravenously normalizes retinal vasopermeability via suppression of the intraocular VEGF level in streptozotocin-induced diabetic rats.21 These reports have indicated that improvement of blood flow by tissue kallikrein may have a potential clinical role in preventing retinal neovascularization. Because tissue kallikrein is used clinically as an orally administered vasodilator for improvement of choroidal blood flow, it may affect retinal neovascularization in diabetic retinopathy. However, to our knowledge, no studies have yet reported a role for tissue kallikrein in the pathogenesis of diabetic retinopathy, an antiangiogenic effect of tissue kallikrein, or the underlying mechanism.

On the basis of the available reports, we measured VEGF and tissue kallikrein in vitreous fluid from patients with PDR to define the relationship between VEGF and tissue kallikrein. Next, we investigated the effects of tissue kallikrein on VEGF165-induced angiogenesis and observed not only the effects of tissue kallikrein against the VEGF165 signaling pathway using human umbilical vein endothelial cells (HUVECs) and human retinal microvascular endothelial cells (HRMECs) but also the cleaving action of tissue kallikrein on
VEGF<sub>165</sub> itself. We also evaluated the antiangiogenic effect of tissue kallikrein by peripheral administration in a murine oxygen-induced retinopathy (OIR) model.

**Methods**

An expanded Methods section is available in Supplemental Data I, available online at http://atvb.ahajournals.org.

**Preparation of Vitreous Fluid Samples**

This study was conducted according to the tenets of the Declaration of Helsinki and was carried out after approval was received from the institutional review committee of Osaka Medical College. Informed consent was obtained from all patients after an explanation of the purpose and procedures of the study.

Vitreous samples were collected from 97 eyes of 97 individuals with diabetes. Details of the patients are shown in the Table.

## Table. Data From Patients With PDR and Other Ocular Diseases

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>MH</th>
<th>ERM</th>
<th>PDR</th>
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<tr>
<td>(No. of patients)</td>
<td>(40)</td>
<td>(20)</td>
<td>(37)</td>
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<tr>
<td>Age (years, mean±SEM)</td>
<td>66.2±1.0</td>
<td>67.3±2.0</td>
<td>56.1±1.9</td>
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<tr>
<td>No. of female patients</td>
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<td>13</td>
<td>18</td>
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<tr>
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<td></td>
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</tr>
<tr>
<td>+ Proliferative membrane</td>
<td>17</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>Alone</td>
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</tr>
<tr>
<td>Vitreous hemorrhage</td>
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<td>+ PVD</td>
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<td>+ CME</td>
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Complications of each patient are shown in clinical findings. PVD indicates posterior vitreous detachment; CME, cystoid macular edema; RD, retinal detachment.

**Results**

**Elevated Tissue Kallikrein and VEGF Concentrations in Vitreous Fluids From PDR Patients**

We measured the concentrations of tissue kallikrein and VEGF in vitreous fluids obtained from patients with PDR and other diseases. The concentration of tissue kallikrein was significantly higher in PDR patients (mean±SEM, 93 821.2±12 192.3 pg/mL, n=37) than in other patients (MH, 15 698.8±3134 pg/mL, n=40; ERM, 20 625.7±3367.9 pg/mL, n=20) (Figure 1A and 1B). Concentrations of VEGF were also higher in PDR patients (787.1±208.4 pg/mL, n=37, versus MH; 4.7±1.1 pg/mL, n=40; ERM; 0.1±0.1 pg/mL, n=20) (Figure 1C). The correlation between concentrations of tissue kallikrein and VEGF in vitreous fluids of all patients was significantly positive (Spearman ρ=0.494, P<0.0001) [Figure 1D].

**VEGF<sub>165</sub>-Induced Tube Formation, Cell Proliferation, and Migration Were Suppressed by Tissue Kallikrein**

HUVECs became organized into complex tubular networks in response to VEGF<sub>165</sub> (10 ng/mL), and this effect was concentration-dependently suppressed by tissue kallikrein (Figure 2A). Tissue kallikrein (0.01 to 10 μg/mL) concentration-dependently inhibited tube formation and significantly inhibited tube area when supplied at 1 and 10 μg/mL (Figure 2B). It also reduced length, joints, and paths when supplied at 0.1 to 10 μg/mL (Figure 2C to 2E).

Cell proliferation in HRMECs was increased to 1.5 times the rate in control cells by VEGF<sub>165</sub> (10 ng/mL) treatment (Figure 2F). Tissue kallikrein inhibited this proliferation; its effect was significant at 10 μg/mL. On the other hand, tissue kallikrein alone had little effect on basal proliferation (Figure 2F). Tissue kallikrein also inhibited the VEGF<sub>165</sub>-induced migration at 1 and 10 μg/mL in HRMECs (Figure 2G and 2H).

**Tissue Kallikrein Suppressed VEGF<sub>165</sub>-Induced Phosphorylation of VEGF Receptor-2, ERK1/2, and p38 Mitogen-Activated Protein Kinase**

The phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) and the phosphorylation of p38 mitogen-activated protein kinase (p38MAPK) are important signaling events for the proliferation and migration of endothelial cells following induction by VEGF. Therefore, we examined the effects of tissue kallikrein on the VEGF<sub>165</sub>-induced phosphorylation of ERK1/2 and p38MAPK in HUVECs. Tissue kallikrein at 10 μg/mL significantly suppressed VEGF<sub>165</sub>-induced ERK1/2 and p38MAPK phosphorylation (Figure 3A).

We next investigated the action of tissue kallikrein on VEGF receptor-2 (VEGFR-2) in the uppermost stream of VEGF<sub>165</sub> signaling by examining the effect of tissue kallikrein on the VEGF<sub>165</sub>-induced VEGFR phosphorylation in HUVECs. VEGF<sub>165</sub> (10 ng/mL) increased the phosphorylation of VEGFR, and tissue kallikrein at 1 and 10 μg/mL significantly inhibited this increase in both HUVECs and HRMECs (Figure 3B and 3C).

**Tissue Kallikrein Cleaved VEGF<sub>165</sub>**

Tissue kallikrein cleaved VEGF<sub>165</sub>, as confirmed by immunoblot analysis with 2 kinds of anti-VEGF antibodies...
The VEGF165 product, following cleavage by tissue kallikrein, could be bound to an anti–N-terminal VEGF antibody but not to an anti–C-terminal VEGF antibody (Figure 4Aa and 4Ab). These results indicate that tissue kallikrein cleaves VEGF165 at the C terminus. Cleavage by tissue kallikrein was concentration dependent. When tissue kallikrein was supplied at 1 μg/mL, intact VEGF165 could still be clearly resolved, whereas at 10 μg/mL, cleaved VEGF165 was clearly detected (Figure 4A). Tissue kallikrein at 10 μg/mL decomposed VEGF165 in a time-dependent manner (Figure 4B). In the vitreous fluids of both MH and PDR patients, the cleaved form of VEGF165 was not detected (Figure 4C). When tissue kallikrein was supplied (at a final concentration of 10 μg/mL), the cleaved form of the VEGF165 was detected (Figure 4C). The cleaved VEGF165 protein was digested with Lys-C to yield peptides that have amino groups at both ends and a C-terminal peptide that has only an ε-amino group. Fragments of C-terminal peptides were not observed (Figure 4Da). From this result, it was supposed that the C-terminal amino acid of the cleaved VEGF165 was a Lys. Mass spectrometry after the Lys-C digestion showed the C-terminal peptides to be CECRPK (iodoacetamide-CECRPK; m/z 849) (Figure 4Db). Theoretical fragments on the C-terminal side were not otherwise observed. On the other hand, although a peak of MSFLQHNKCE (iodoacetamide-MSFLQHNKCE; m/z 1293) was observed following Glu-C digestion, theoretical fragments on the C-terminal side were not detected, as for Lys-C digestion (Figure 4Dc). These results indicate that the C-terminal amino acid residue of VEGF165 cleaved by tissue kallikrein is Lys (Lys107, Lys108, or both) (Figure 4E).

Tissue Kallikrein Inhibited Retinal Neovascularization in a Murine OIR Model

The effect of tissue kallikrein on retinal neovascularization in vivo was evaluated using an OIR model. Tissue kallikrein significantly decreased the retinal neovascularization compared with vehicle treatment, which was observed as abnormal retinal vessel formation by fluorescein conjugated dextran (Figure 5Aa, 5Ac, 5Ae, 5Ag, and 5Ai). The node regions (represented by green labels in the analyzed image) were decreased by tissue kallikrein treatment (versus vehicle) (Figure 5Ab, 5Ad, 5Af, 5Ah, and 5Aj). The tissue kallikrein–treated group was significantly suppressed (versus vehicle) in both the number of nodes and the nodes area in a dose-dependent manner (Figure 5B and 5C). Side effects of tissue kallikrein were checked in normal retinal vessels by measuring the capillary-free area, which was caused by centering on optic nerve (Figure 5Db and 5Dc). Revascularization of the capillary-free area did not differ between vehicle and tissue kallikrein treatments (Figure 5E). In tissue kallikrein–treated group, we detected cleaved VEGF164 and a decrease in VEGF164 (Figure 5F).

Discussion

In the present study, we confirmed that the vitreous concentrations of tissue kallikrein and VEGF were higher in PDR patients than in control patients with MH and ERM. We also found a positive correlation between tissue kallikrein and VEGF (in the entire patient contingent). Tissue kallikrein inhibited VEGF165-induced tube formation, proliferation, and migration in our in vitro model and also cleaved VEGF165. In the in vivo study, the systemic administration of tissue kallikrein inhibited the formation of abnormal retinal vessels in OIR model mice without affecting normal vessels.
To elucidate the correlation of tissue kallikrein and VEGF, we measured their concentrations in the vitreous fluids of patients with PDR, MH, and ERM. Tissue kallikrein was not only significantly higher in PDR patients than the control patients, but tissue kallikrein and VEGF also showed a positive correlation with each other. These findings indicate that an increase in tissue kallikrein might prevent the abnormal increase in VEGF in PDR patients and therefore might be efficacious for retinal neovascularization.

In the present in vitro study, tissue kallikrein at 0.1 to 10 μg/mL inhibited VEGF165-induced tube formation (tube length, joints, and paths) and both VEGF165-induced proliferation and migration in HUVECs (Supplemental Figure I) and HRMECs. These data indicate that tissue kallikrein inhibits VEGF165-induced tube formation through the suppression of both proliferation and migration of endothelial cells.

The VEGF165-induced phosphorylation of ERK1/2, an important factor involved in VEGF165-induced endothelial cell proliferation,23 is also inhibited in the presence of bradykinin.24 In a HRMEC proliferation test, a dual antagonist of bradykinin B₁ and B₂ had no effect on the antiproliferative activity of tissue kallikrein (Supplemental Figure II), indicating that tissue kallikrein might suppress the VEGF165-induced proliferation independently of bradykinin receptors.

Next, we studied the mechanism of antiangiogenic effect of tissue kallikrein using immunoblot analyses for VEGF signaling. VEGF signaling includes both ERK1/2 and p38MAPK, which are primarily involved in cell proliferation and migration.23,25–27 In the present study, tissue kallikrein inhibited VEGF165-induced angiogenesis by suppression of cell proliferation and migration; its mechanism might be due to inhibition of the ERK1/2 and p38MAPK pathways via...
inhibition of the phosphorylation of VEGFR-2, which exists in the uppermost stream of VEGF signaling.

To clarify the mechanism of antiangiogenic effects of tissue kallikrein, we investigated whether tissue kallikrein has a direct action on VEGF. VEGF165 exists at high frequency, and its physiological activity is high compared with other isoforms. Although tissue kallikrein cleaves Met-Lys and Arg-Ser bonds in kininogen, we identified that VEGF165 was interestingly cleaved at its C terminus (Lys107, Lys108, or both) by tissue kallikrein. The heparin binding function of VEGF165 reportedly is completely mediated by the C-terminal domain. Therefore, the cleaved VEGF165 product cut by tissue kallikrein appeared to lose almost all of the bioactivity for VEGFR-2. In the present experiments, tissue kallikrein at 10 μg/mL inhibited VEGF165-induced proliferation and migration; thus, the cleavage effect of tissue kallikrein at 0.1 to 10 μg/mL for VEGF165 (10 ng/mL) was consistent with the concentrations (0.1 to 10 μg/mL) in our in vitro studies (VEGF165-induced tube formation, proliferation, and migration). These concentrations in both the tube formation and the cleavage assay were consistent with those in the human vitreous with PDR. These findings indicate that tissue kallikrein at 100 ng/mL (a concentration in the human vitreous) might have an antiangiogenic effect via the degradation of VEGF165.

We investigated the antiangiogenic effect of peripherally administered tissue kallikrein on retinal neovascularization using OIR model mice as an in vivo model. The systemic administration of tissue kallikrein inhibited retinal neovascularization, although tissue kallikrein had no influence on the size of the capillary-free area (an index of retinal revascularization), on behavior, or on body weight. We confirmed that tissue kallikrein reduced the expression of VEGF164 (termed VEGF165 in humans) in the retina of the OIR model mice.
Figure 4. Cleavage action on VEGF₁₆₅ by tissue kallikrein. A. After being incubated for 6 hours with tissue kallikrein (0.1 to 10 μg/mL), the digestion products of VEGF₁₆₅ (10 ng/mL) were analyzed by immunoblotting using both anti-N-terminal VEGF antibody (a) and anti-C-terminal VEGF antibody (b). B. Digested VEGF₁₆₅ (10 ng/mL) incubated with tissue kallikrein (10 μg/mL) for 0 to 6 hours was analyzed by immunoblotting using an anti-N-terminal VEGF antibody. C. To confirm the cleaved VEGF₁₆₅ by using human vitreous fluids, we demonstrated immunoprecipitation (IP) of VEGF₁₆₅ with vitreous fluids in MH and PDR patients. D. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra after tris(trimethoxyphenyl) phosphonium acetyl (TMPP-Ac) modification of peptides digested by Lys-C (a). Mass spectrometry (MS) spectra results after the Lys-C digestion (b) and Glu-C (c) are indicated. The x- and y-axes represent m/z and percentage intensity, respectively, for all mass spectra. E. The theoretical fragments on the C-terminal side were not detected beyond Lys108. The C-terminal amino acid of VEGF₁₆₅ cleaved by tissue kallikrein was Lys (Lys107, Lys108, or both). Cont indicates control.
Similar to the in vitro study, tissue kallikrein at a concentration that inhibits retinal neovascularization in the OIR model might decompose. In a tissue kallikrein–treated group, the cleaved form of the VEGF164 and a decrease in VEGF164 were detected. The data indicate that tissue kallikrein inhibited retinal neovascularization via the cleavage of VEGF164. We have reported that tissue kallikrein decreased the expression of VEGF164 by upregulating the production of nitric oxide.21 Taken together, these data suggested that tissue kallikrein might inhibit retinal neovascularization via the dual action of both cleaving the VEGF164 and decreasing the expression of VEGF in the OIR model.

In newborn mouse pups, the primary vascular network had reached the periphery by approximately P8.33 At the termination of the experiment (P8), there were no statistically significant differences in any parameters (tube area, length, joints, and paths) between control and the tissue kallikrein treatment (at a dosage of 50 µg/kg) (Supplemental Figure IV). These data strongly suggested that tissue kallikrein did not inhibit retinal physiological angiogenesis. Retinal physiological vascular development required VEGF121 (termed VEGF120 in the mouse), whereas VEGF165 (termed VEGF164 in the mouse) was deeply involved in retinal pathological angiogenesis.34,35 Tissue kallikrein did not inhibit either
proliferation or migration induced by VEGF<sub>121</sub> (Supplemental Figure VA and VB). Tissue kallikrein had no effect on the activity of VEGF<sub>165</sub> (Supplemental Figure VC). For selective cleavage of VEGF<sub>165</sub>, tissue kallikrein might increase to maintain homeostasis in human vitreous fluids.

In conclusion, tissue kallikrein might be partly involved in the pathogenesis of PDR, and it may have an antiangiogenic effect through the cleavage of VEGF<sub>165</sub> selectively when administered by a peripheral route. Our findings indicate the clinical possibilities of tissue kallikrein application to be as follows: (1) the antiangiogenic mechanism of tissue kallikrein is different from the remedies used in the retinal neovascularization, and (2) the administration route is peripheral, with a low risk of side effects and a high compliance compared with therapeutic methods using intravitreal administration. Although tissue kallikrein has been used clinically as an oral agent for 22 years, no side effects, such as inhibiting normal neovascularization, have been reported at all. Therefore, tissue kallikrein may be a promising therapeutic agent for retinal neovascularization or hyperpermeability in patients with PDR, age-related macular degeneration, central retinal vein occlusion, and neovascular glaucoma.

**Disclosures**

None.

**References**

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A

Supplementary figure. I

B

C

Proliferation rate (fold increase)

Cont Veh 0.1 1.0 10 µg/ml

KAL

VEGF₁₆₅

Migration rate (fold increase)

Cont Veh 0.1 1.0 10 µg/ml

KAL

VEGF₁₆₅
Supplementary figure. II

2.1

1.5

N.S.

a
t (fold increase)

0

0.5

Cont

KAL

1

10

Veh

0.1

1

10 µM

Proliferation rate (fold increase)

BK antagonist

BK antagonist

KAL (10 µg/mL)

VEGF\textsubscript{165}
Supplementary figure. III

1.5

N.S.

Density total-VEGFRs/β-actin (fold increase)

Cont  Veh  1  10  10 µg/ml

KAL

VEGF_{165}
Supplementary figure. V

A

Proliferation rate (fold increase)

Cont  Veh  0.1  1  10  10 µg/ml

KAL

N.S.

B

Migration rate (fold increase)

Cont  Veh  0.1  1  10  10 µg/ml

KAL

N.S.

C

D

VEGF_{121}

Cont  Veh  KAL (10 µg/ml)

p-VEGFR

Total-VEGFR

β-actin

N.S.

Density p-VEGFRs / total VEGFRs (fold increase)

Veh  KAL (10 µg/ml)

VEGF_{121}
Supplements

Supplement 1:

Methods

Purification procedure of tissue kallikrein

Rat kallikrein was purified from urine to apparent homogeneity by ultrafiltration and ion-exchange chromatography on a DEAE-Sepharose FF column (GE Healthcare, Buckinghamshire, UK), affinity chromatography on an aprotinin (Sigma, MO, USA) coupled HiTrap NHS-activated Sepharose HP column (GE Healthcare) and gel filtration twice on a TSK gel G3000SXXL column (Tosoh Corporation, Tokyo, Japan) according to the modified methods.1, 2 The enzyme preparation migrated as a single band with an apparent molecular mass of 33 kDa on SDS-PAGE under reducing conditions and eluted as a single peak with an apparent molecular mass of 53 KDa on gel filtration chromatography. The purified rat urine kallikrein had a specific activity of 47.619 µmol/min/mg protein towards the synthetic substrate Pro-Phe-Arg-MCA (Peptide Institute, Osaka, Japan).

Measurement of tissue kallikrein and VEGF
The levels of tissue kallikrein in the vitreous body from patients with MH, ERM, and PDR were measured by Western blot analysis. Anti-human tissue kallikrein (kallikrein) antibody (Sanwa Kagaku Kenkyusho Co., Ltd., Mie, Japan) which was pre-absorbed with human plasma kallikrein (R&D Systems, Inc., MN, USA) was used as primary antibody and human urine tissue kallikrein (Sanwa Kagaku Kenkyusho Co., Ltd.) was used as a standard. Samples were separated on a 4-12 % polyacrylamide bis-tris gel (Invitrogen, CA, USA) and transferred to PVDF membrane (Invitrogen). The membrane was blocked with 5% skim milk in Tris Buffered Saline with Tween 20 (TBST) for 1 hour at room temperature and incubated with a 1:2000 dilution of primary antibody overnight at 4 °C. The membrane was washed with TBST and incubated with a 1:5000 dilution of horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (GE Healthcare, Buckinghamshire, UK) for 2 hours at room temperature. Tissue kallikrein was visualized using an enhanced chemiluminescence (ECL) plus western blotting detection system (GE Healthcare) and Cool Saver (Atto, Tokyo, Japan). Densitometric quantifications were performed using Cs Analyzer software (Atto). The levels of tissue kallikrein were calculated with reference to the standard curve.

Quantitative VEGF determination
The VEGF concentration in the vitreous body from patients with MH, ERM, and PDR were measured by the AlphaLISA VEGF kit (PerkinElmer Life and Analytical Sciences, Boston, MA, USA) according to the manufacturer's instructions. For statistical analysis, any VEGF level (2.2 pg/ml) below the limit of detection was set to zero.

Briefly, 5 µl of samples and standards were added to a white 96-well half area plate (PerkinElmer Life and Analytical Sciences). Then, 10 µl of 5 × alphaLISA anti-VEGF acceptor beads were added to the plate and incubated for 30 minutes at 23 °C. A 10 µl volume of 5 × biotinylated anti-VEGF antibody was added to the plate and incubated for 60 minutes at 23 °C. Finally, 25 µl of 2 × streptavidin Donor beads were added to the plate and incubated for 30 minutes at 23 °C in the dark. The plate was scanned with a Power scan 4 Multi-Mode Microplate Reader (DS Pharma Biomedical, Osaka, Japan) and the data were analyzed using Gen5 Data Analysis Software (BioTek instruments, Winooski, VT).

Cell culture

Primary human retinal microvascular endothelial cells (HRMECs) were obtained from DS Pharma Biomedical (Osaka, Japan) and cultured in CS-C medium and culture
boost (growth factors) at 37 °C in a humidified atmosphere of 5% CO2 in air.

**Tube formation assay**

An angiogenesis assay kit (Kurabo) was used according to the manufacturer’s instructions. HUVECs co-cultured with fibroblasts were cultivated in the presence or absence of various concentrations of tissue kallikrein [purified from rat urine (Sanwa Kagaku Kenkyusho Co., Ltd., Japan)] plus VEGF<sub>165</sub> (10 ng/ml) at days 1, 4, 7, and 9. At day 11, cells were fixed in 70% ethanol. The cells were incubated with diluted primary antibody (mouse anti-human CD31, 1 : 4000) for 1 h at 37 °C, and with the secondary antibody (goat anti-mouse IgG alkaline phosphatase-conjugated antibody, 1 : 500) for 1 h at 37 °C, and visualization was achieved using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium. Images were obtained from five different fields (5.5mm<sup>2</sup> per field) for each well, and tube area, length, joints, and paths were quantified using Angiogenesis Image Analyzer Ver.2 (Kurabo).

**Cell proliferation**

HRMECs were seeded into 96 well plates at a density 2×10<sup>3</sup> cells per well at 37°C for 24 h. HUVECs were rinsed with phosphate buffered saline (PBS) (137 mM
sodium chloride, 2.7 mM potassium chloride, 10.1 mM disodium hydrogen phosphate 12 hydrate, and 1.8 mM potassium dihydrogenphosphate), and were preincubated in HuMedia-EB2 containing 2% FBS at 37°C for 24 h. HRMECs were rinsed with PBS, then exposed for 12 h to CS-C medium containing 10% FBS without cell boost. HUVECs or HRMECs were incubated for 72 h or 24 h in fresh medium containing VEGF165 (10 ng/ml) with or without various concentrations of tissue kallikrein. After incubation, the viable cell rate was measured by means of a WST-8 assay. Briefly, 10 µl of CCK-8 (Dojindo, Kumamoto, Japan) was added to each well, incubated at 37°C for 3 h, and the absorbance measured at 492nm.

**Wound-healing assay**

A wound-healing assay was performed to measure unidirectional migration by HUVECs or HRMECs. HUVECs or HRMECs were seeded at $4 \times 10^4$ cells/well into a 12 well plate, incubated for 48 h at 37°C in a humidified atmosphere of 5% CO₂, then washed with PBS and incubated in Humedia-EB2 or CS-C medium with 1% FBS. After 24 h incubation, the monolayers of HUVECs or HRMECs were scratched to a 1 mm depth in a straight line using a 10-200 µl micro-tip. For stimulation, VEGF165 (10 ng/ml) and/or tissue kallikrein were added, and incubation was continued for 24 h.
Images were taken at the time of the wounding and at 24 h intervals thereafter using a phase-contrast microscope (Olympus, Tokyo, Japan). Migration was estimated by counting the cell numbers within the wounded region. Invading cells were counted in a masked fashion by a single observer (S. N.), and taken as migrating cells. For each monolayer sample, four measurements were taken from four fields in each of three independent wounds.

Immunoblot analysis

Subconfluent HUVECs or HRMECs were incubated in HuMedia-EB2 or CS-C medium containing 2% FBS for 24 h at 37°C in a 5% CO2 atmosphere. The medium was then changed to Dulbecco’s modified Eagle medium containing 25 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (Invitrogen, Grand Island, NY, USA) and either 2% FBS for VEGF receptor-2 (VEGFR-2), extracellular-regulated kinase (ERK) and p38 mitogen-activated protein kinase (p38) MAPK detection, and incubation allowed to proceed for a further 6 h at 37°C. The medium was then changed to fresh medium (constituents as above) containing VEGF165 (10 ng/ml) with or without tissue kallikrein, and incubation continued for 2, 5 or 10 min (we performed a pilot study for time course of changes in phosphorylated–VEGFR-2, ERK 1/2, and
p38MAPK after VEGF treatment). The HUVECs were washed twice in PBS, lysed in RIPA buffer (Sigma, St. Louis, MO, USA) supplemented with protease inhibitor cocktail (Sigma), phosphatase inhibitor cocktail 1 (Sigma), and phosphatase inhibitor cocktail 2 (Sigma), and stocked at -80°C.

Equal amount of each sample were electrophoresed on 10% SDS-PAGE gel, then transferred to PVDF membranes. After blocking with Blocking One-P (Nacarai tesque, Kyoto, Japan) for 30 min, the membranes were incubated with one of the following, as the primary antibody: anti-phosphorylated VEGFR-2 (Cell Signaling Technology, Beverly, MA, USA), anti-total VEGFR-2 (Cell Signaling Technology), anti-phosphorylated ERK 1/2 (Cell Signaling Technology), anti-total ERK 1/2 (Cell Signaling Technology), anti-phosphorylated p38MAPK (Promega, Madison, WI, USA), anti-total p38MAPK (Promega), or anti β-actin antibody (Sigma). After incubation, the membrane was incubated with secondary antibody: HRP conjugated goat anti-rabbit or -mouse IgG (Pierce Biotechnology, Rockford, IL, USA). The immunoreactive bands were visualized using Super Signal® West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific K.K., Waltham, MA, USA) and measured using LAS-4000 mini (Fujifilm, Tokyo, Japan).
Cleavage assay

VEGF\textsubscript{165} (R&D Systems, Minneapolis, MN, USA) was incubated in CS-C medium with or without tissue kallikrein at the desired concentration. To investigate concentration- and time-dependent drug effects, VEGF\textsubscript{165} (10 ng/ml) was incubated in CS-C medium containing 0.5% FBS for 0 to 6 h at 37°C with or without tissue kallikrein (0.1 to 10 µg/ml). Equal amounts of each sample were electrophoresed on 15% SDS-PAGE gel, then subjected to immunoblot analysis using primary anti-bodies: anti N-terminal VEGF antibody (Thermo Fisher Scientific Inc., Waltham, MA, USA) and anti C-terminal VEGF antibody (Epitomics Inc., Burlingame, CA, USA). After electrophoresis, the SDS-PAGE gels were rinsed three times with deionized water for 5 min in a staining tray, and then drained. The gels were covered with Quick-CBB PLUS (Wako, Osaka, Japan) in the tray and shaken for 60 min until the protein bands were visible. When we performed the cleavage assay using human vitreous fluids, five samples from MH and PDR were collected to detect the cleaved form of the VEGF. These samples were incubated for 6 h at 37°C with or without tissue kallikrein (final concentration 10 µg/ml). VEGF in vitreous fluids was concentrated by immunoprecipitation with the Pierce® Classic IP Kit with anti-VEGF antibody, as the designated protocol. After immunoprecipitation, immunoblot analysis was performed
with anti-VEGF antibody.

In vivo, mice were euthanatized using sodium pentobarbital at 80 mg/kg, i.p., and their eyeballs were quickly removed. The retinas were quickly frozen in dry ice. Next, their cornea and lens were removed. Furthermore, we extracted both retina and intravitreous fluids. For protein extraction, the tissue was homogenized in cell-lysis buffer using a homogenizer (Phycotron; Microtec Co. Ltd., Chiba, Japan). We performed immunoprecipitation and immunoblot analysis as well as human vitreous fluids. Tissue kallikrein was given subcutaneously at 50 µg/kg once a day, immediately after hyperoxia (P12) until P16. Six eyes from three animals in each group were enucleated.

C-terminal sequence analysis

The cleaved VEGF<sub>165</sub> was separated by SDS-PAGE in a 15% polyacrylamide gel. The Coomassie-stained protein band was excised and washed with 50% v/v acetonitrile (Wako) in 100 mM NaHCO<sub>3</sub>. The washed gel piece was dehydrated with acetonitrile and dried in a vacuum centrifuge. To the dried gel 100 mL of 10 mM aqueous tris (2-carboxyethyl) phosphine hydrochloride (TCEP, Fluka, Switzerland) solution was added to reduce disulfide bonds. This solution was incubated for 30 min at 37°C.
S-alkylation was accomplished by replacing the TCEP solution with 55 mM iodoacetamide in 100 mM NaHCO₃. After 45 min incubation at room temperature in the dark, the gel piece was washed with 100 mL of 50 mM NaHCO₃, dehydrated in acetonitrile, and dried in a vacuum centrifuge. The gel piece was then rehydrated with 2 mL of acetonitrile:50 mM NaHCO₃ (1:9, v/v) containing 200 ng of Lys-C. After 5 min, 50 mM NaHCO₃ (15 mL) was added to keep the gel piece moist during digestion (37°C, overnight). To extract the resulting peptides, 30 mL of 50% acetonitrile containing 0.05% trifluoroacetic acid (Wako) was added to the digestion mixture, and the gel piece was sonicated in a water bath for 10 min, after which the supernatant was collected. This extraction procedure was repeated three times. The extracts were combined and lyophilized. The resulting powder was dissolved in 10 mL of acetonitrile-50 mM NaHCO₃ (1:9). To this solution 1 mL of succinimidyloxycarbonylmethyl tris (2,4,6-tri-methoxyphenyl) phosphonium bromide (TMPP-Ac-OSu, Fluka, Switzerland) (10 mM in acetonitrile-water, 1:4, v/v) was added and the mixture was sonicated in a water bath for 30 min. The TMPP-modified solution was added to the prewashed p-phenylenediisothiocyanate resin (DITC, Shimadzu Corporation, Kyoto, Japan), and this was allowed to stand for 2 h in a water bath at 60°C. After extraction with acetonitrile-50 mM NaHCO₃ (1:9, 60 mL; twice)
and 2-propanol-acetonitrile-0.1% trifluoroacetic acid (1:1:2, 60 mL; three times), the extracts were combined and dried in a vacuum centrifuge.

**Enzyme digestion and mass spectrometry**

The gel piece was washed with water while vortexing and dehydrated with acetonitrile. Acetonitrile was removed and the gel piece was dried in a vacuum centrifuge. Ten mM dithiotreitol in 100 mM ammonium bicarbonate was added and the solution was incubated for 1 hr at 56°C. The solution was replaced by the same volume of 55 mM iodoacetamide in 100 mM ammonium bicarbonate and incubated for 45 min at room temperature in the dark with vortexing. The gel piece was washed with 100 mM ammonium bicarbonate while vortexing, then dehydrated with acetonitrile and rehydrated again with 100 mM ammonium bicarbonate and dehydrated again. Acetonitrile was removed and the gel pieces were dried in a vacuum centrifuge. Lys-C or Glu-C solution (50 mM ammonium bicarbonate, 5 mM calcium chloride, 10 µg/ml Lys-C or Glu-C) was added and the solution was incubated for 45 min at 4°C. After incubation at 4°C, the solution was incubated for at 25°C for 12 h. Peptides treated with Lys-C or Glu-C were extracted using sequential steps of 20 mM ammonium bicarbonate, followed by 5% formic acid in 50% acetonitrile. The combined extract
was dried in a vacuum centrifuge. Trifluoroacetic acid (0.1%) was added to the residue and the solution was desalted using ZipTip$_{µ}$-C$_{18}$ (Millipore Corp., Billerica, MA, USA) according to the manufacturer's protocol. The peptides were eluted with trifluoroacetic acid in 50% acetonitrile and applied to a MALDI plate. Mass spectrometry of peptides was performed using MALDI-TOF MS (AXIMA-Performance; Shimadzu). All measurements were performed in positive-ion reflection mode.

Animals

C57BL/6 mice (SLC, Shizuoka, Japan) were used. All investigations were in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research, and the experiments were approved and monitored by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University.

Visualization of the retinal flat-mount by angiography and its quantification

Mice were deeply anesthetized intraperitoneally with sodium pentobarbital (Nembutal; Dainippon-Sumitomo Pharmaceutical Co. Ltd., Osaka, Japan) at 30 mg/kg. Through a median sternotomy, they were perfused through the left ventricle with
fluorescein-conjugated dextran (Sigma) dissolved in PBS. The eyes were then enucleated and placed in 4% paraformaldehyde. Under a microscope, the cornea and lens were removed from each eye, and the retinas were dissected, flat-mounted and covered with a coverslip after a few drops of Fluoromount™ aqueous mounting medium for fluorescent staining (Diagnostic BioSystems, Pleasanton, CA, USA) had been placed on the slide. We used a method for measuring retinal neovascularization using imaging software as reported previously. To evaluate pathological neovascularization, quantification of the retinal vasculature was performed using the Angiogenesis Tube Formation module in Metamorph (Universal Imaging Corp., Downingtown, PA, USA). We evaluated the number of nodes and node areas, which are parameters that are obtained from these analyzed images. The node is the region of the connected ‘blobs’ with thickness exceeding maximum width of the vessels and means the region where there is pooling of fluorescein conjugated dextran. These regions are shown as green labels in analyzed images and corresponded well to the pathological neovascularization area (including tortuous and dilated blood vessels, and abnormal vascular structure). To evaluate the capillary-free area, retinal revascularization was measured.
Statistical analysis

Statistical analyses of *in vitro* and *in vivo* experiments were performed with the aid of the Statistical Package for the Social Sciences 15.0J for Windows software (SPSS Japan Inc, Tokyo, Japan). Data are presented as mean ± SEM for the *in vitro* and *in vivo* studies. Statistical comparisons of *in vitro* and *in vivo* experiments were made using a one-way ANOVA followed by Student’s *t*-test or Dunnet’s multiple comparison test. Statistical comparisons of clinical samples were made using Steel Dwass’s multiple-comparison test and Spearman’s correlation test (R-2.8.1 for Windows, ISBN 3-900051-07-0, URL http://www.R-project.org.). A value of < 0.05 was considered to indicate statistical significance.
Supplementary data

Methods

Cell proliferation and migration in HUVECs

Human umbilical vein endothelial cells (HUVECs, Kurabo, Osaka, Japan) were cultured in a growth medium (HuMedia-EG2; Kurabo) at 37 °C in a humidified atmosphere of 5% CO2 in air. The HuMedia-EG2 medium consists of a base medium (HuMedia-EB2, Kurabo) supplemented with 2% fetal bovine serum (FBS), 10 ng/ml recombinant human epidermal growth factor (hEGF), 1 µg/ml hydrocortisone, 50 µg/ml gentamicin, 50 ng/ml amphotericin B, 5 ng/ml recombinant human basic fibroblast growth factor-B (hFGF-B) and 10 µg/ml heparin. Subconfluent monolayers of HUVECs, from passages 3 to 7, were used in the experiments.

HUVECs were seeded into 96 well plates at a density 2×10^3 cells per well at 37°C for 24 h. HUVECs were rinsed with phosphate buffered saline (PBS) (137 mM sodium chloride, 2.7 mM potassium chloride, 10.1 mM disodium hydrogen phosphate 12 hydrate, and 1.8 mM potassium dihydrogenphosphate), and were preincubated in HuMedia-EB2 containing 2% FBS at 37°C for 24 h. Moreover, we examined in the same way by using HRMECs. The migration assay for HUVECs was performed in the
same way as described for HRMECs.

**Retinal physiological angiogenesis model in mice**

C57BL/6 mice (SLC, Shizuoka, Japan) were mated and we used neonatal mice. To visualize the retinal blood vessels, they were sampled as described in the ‘Visualization of the retinal flat-mount by angiography and its quantification’ methods section. Tissue kallikrein was given subcutaneously at 50 µg/kg once a day from P3 to P7. The number of doses and the administration route of tissue kallikrein were performed as described for the OIR model. To evaluate retinal physiological angiogenesis, tube area, length, joints, and paths of retinal blood vessels were quantified using the Angiogenesis Tube Formation module in ‘Metamorph.

**Cell proliferation, migration, and the phosphorylation of VEGFR-2 induced by VEGF$_{121}$ in HRMECs**

We examined the cell proliferation, migration, and the phosphorylation of VEGFR-2 induced by VEGF$_{121}$ (R&D Systems), using the procedure described above.
Supplementary figure I. Effect of tissue kallikrein on VEGF_{165}-induced proliferation and migration

HUVECs (A) were cultured in a 96-well plate, and proliferation rates were measured by WST-8 assay. Data are shown as mean ± SEM (n = 6). Images of wounded monolayer of or HUVECs (B) taken at 24 h after treatment with VEGF_{165} (10 ng/ml) with or without tissue kallikrein. (C) Migration was estimated by measurement of cell numbers within the wounded region. Scale bar = 500 µm. Data are shown as mean ± SEM (n = 3 or 4). *, P < 0.05 versus vehicle (Dunnett’s multiple-comparison test). ##, P < 0.01 versus control (Student’s t-test). Cont; Control. Veh; Vehicle. KAL; Tissue kallikrein.

Supplementary figure II. Effect of a bradykinin antagonist on VEGF_{165}-induced HRMEC proliferation.

HRMECs were cultured in a 96-well plate (at a density of 2×10³ cells/well), and incubated for 72 h or 24 h at 37 °C in 5% CO₂. These were supplemented with VEGF_{165} (10 ng/ml) and tissue kallikrein (10 µg/ml) plus various concentrations of bradykinin antagonist, and measurements were made by a WST-8 assay. The bradykinin antagonist had no effect against antiproliferative activity of tissue kallikrein.
Data are shown as mean ± SEM (n = 6). *, P < 0.05 versus control (Dunnett’s multiple-comparison test). ##, P < 0.01 versus control (Student’s t-test). Cont; Control. Veh; Vehicle. KAL; Tissue kallikrein. BK; Bradykinin.

Supplementary figure III. Effect of tissue kallikrein for VEGFR itself.
Quantitative analysis of western blotting of total VEGFR. The data of figure 3C was analyzed. There were no significant deference between vehicle and tissue kallikrein treated group. Data are shown as mean ± SEM (n = 5). Cont : Control. Veh : Vehicle. KAL; Tissue kallikrein.

Supplementary figure IV. Effect of tissue kallikrein on the physiological angiogenesis of mouse.
Representative images show the retinal flat-mount at P0 (A-a) and at P4 (A-b). Shown are retinal blood vessels of the control group (A-c) and of tissue kallikrein treated group (A-d) at P8. Scale bar = 500 µm (A-d). Quantitative analysis of the stained tube-like structures was performed (using an angiogenesis imaging analyzer) in five different fields for each well, measurements being made of tube area (B), length (C), joints (D), and paths (E). Data are shown as mean ± SEM (n = 5). Cont; Control. KAL;
Tissue kallikrein.

Supplementary figure V. Effect of tissue kallikrein on VEGF$_{121}$-induced proliferation, migration, and the phosphorylation of VEGFR-2.

HRMECs (A) were cultured in a 96-well plate, and proliferation rates induced by VEGF$_{121}$ were measured by WST-8 assay. Data are shown as mean ± SEM (n = 6).

Images of wounded monolayer of or HRMECs (B) taken at 24 h after treatment with VEGF$_{121}$ (10 ng/ml) with or without tissue kallikrein. (C) Migration was estimated by measurement of cell numbers within the wounded region. Scale bar = 500 µm. Data are shown as mean ± SEM (n = 3). Tissue kallikrein (10 µg/ml) did not inhibit VEGFR-2 phosphorylation in HRMECs (D) induced by VEGF$_{121}$ (10 ng/ml). Data are shown as mean ± SEM (n = 5).
References

