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 VEGF₁₆₅ (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 (5.5mm² 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 2×10³ 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 VEGF_{165} (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 × 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, VEGF_{165} (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_{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_{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 \mu g/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 \mu g/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 (Physcotron; 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$_{165}$ 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$_3$. 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$_{\mu}$-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 \textit{in vitro} and \textit{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 \textit{in vitro} and \textit{in vivo} studies. Statistical comparisons of \textit{in vitro} and \textit{in vivo} experiments were made using a one-way ANOVA followed by Student’s \textit{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 2×10³ 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 VEGF₁₂₁ in HRMECs**

We examined the cell proliferation, migration, and the phosphorylation of VEGFR-2 induced by VEGF₁₂₁ (R&D Systems), using the procedure described above.
Supplementary figure I. Effect of tissue kallikrein on VEGF₁₆₅-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 VEGF₁₆₅ (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 VEGF₁₆₅-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 VEGF₁₆₅ (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;
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

