ANIMAL MODELS

Adrenomedullin Suppresses Vascular Endothelial Growth Factor—Induced Vascular Hyperpermeability and Inflammation in Retinopathy

Akira Imai,^{*†} Yuichi Toriyama,^{*†} Yasuhiro Iesato,^{*†} Kazutaka Hirabayashi,^{*†} Takayuki Sakurai,^{*} Akiko Kamiyoshi,^{*} Yuka Ichikawa-Shindo,^{*} Hisaka Kawate,^{*} Megumu Tanaka,^{*} Tian Liu,^{*} Xian Xian,^{*} Liuyu Zhai,^{*} Kun Dai,^{*} Keiya Tanimura,^{*} Teng Liu,^{*} Nanqi Cui,^{*} Akihiro Yamauchi,[‡] Toshinori Murata,[†] and Takayuki Shindo^{*}

From the Department of Cardiovascular Research,^{*} Shinshu University Graduate School of Medicine, Nagano; the Department of Ophthalmology,[†] Shinshu University School of Medicine, Nagano; and the Research Division,[‡] Japan Bio Products Co., Ltd., Tokyo, Japan

Accepted for publication
January 19, 2017.

Address correspondence to
Takayuki Shindo, M.D., Ph.D.,
Department of Cardiovascular
Research, Shinshu University
Graduate School of Medicine,
Asahi 3-1-1, Matsumoto, Nagano
390-8621, Japan. E-mail:
tshindo@shinshu-u.ac.jp.

Diabetic macular edema (DME) is caused by blood-retinal barrier breakdown associated with retinal vascular hyperpermeability and inflammation, and it is the major cause of visual dysfunction in diabetic retinopathy. Adrenomedullin (ADM) is an endogenous peptide first identified as a strong vasodilator. ADM is expressed in the eyes and is up-regulated in various eye diseases, although the pathophysiological significance is largely unknown. We investigated the effect of ADM on DME. In Kimba mice, which overexpress human vascular endothelial growth factor in their retinas, the capillary dropout, vascular leakage, and vascular fragility characteristic of diabetic retinopathy were observed. Intravitreal or systemic administration of ADM to Kimba mice ameliorated both the capillary dropout and vascular leakage. Evaluation of the transendothelial electrical resistance and fluorescein isothiocyanate-dextran permeability of an endothelial cell monolayer using TR-iBRB retinal capillary endothelial cells revealed that vascular endothelial growth factor enhanced vascular permeability but that co-administration of ADM suppressed the effect, in part by enhancing tight junction formation between endothelial cells. In addition, a comprehensive PCR array analysis showed that ADM administration suppressed various molecules related to inflammation and NF- κ B signaling within retinas. From these results, we suggest that by exerting inhibitory effects on retinal inflammation, vascular permeability, and blood-retinal barrier breakdown, ADM could serve as a novel therapeutic agent for the treatment of DME. (*Am J Pathol* 2017, 187: 999–1015; <http://dx.doi.org/10.1016/j.ajpath.2017.01.014>)

Diabetic retinopathy (DR) is the leading cause of blindness among adults in developed countries. DR is considered to be a microvascular complication caused by a vicious cycle initiated by hyperglycemia. Within the retina, prolonged hyperglycemia enhances the production and accumulation

of advanced glycation end products and evokes chronic inflammation, elevated oxidative stress, hypoxia, and vascular hyperpermeability. To determine the best approach to treatment and to assess prognosis, DR is divided into two stages: nonproliferative DR (NPDR; subdivided into mild,

Supported by a grant-in-aid for scientific research (KAKENHI), Core Research for Evolutionary Science and Technology (CREST) of Japan Science and Technology Agency (JST) and the Japan Agency for Medical Research and Development (AMED) (T.Sh.); a National Cardiovascular Center research grant for cardiovascular diseases (T.Sh.); a grant-in aid of The Public Trust Fund For Clinical Cancer Research, Mitsui Life Social Welfare Foundation (T.Sh.); a Takeda Science Foundation research grant (T.Sh.); an Opto-Science and Technology research grant (T.Sh.); a Takeda Medical Research Foundation grant (T.Sh.); an Elderly Eye Disease

Research Foundation grant (T.Sh.); a Novartis Foundation for Gerontological Research grant (T.Sh.); research grants from the Cosmetology Research Foundation (T.Sh.), SENSHIN Medical Research Foundation (T.Sh.), Kanzawa Medical Research Foundation (T.Sh.), Ono Medical Research Foundation (T.Sh.), Nagao Memorial Fund (T.Sh.), Nakatomi Foundation (T.Sh.), and Japan Vascular Disease Research Foundation (T.Sh.); YOKOYAMA Foundation for Clinical Pharmacology grant (Y.Ie.); and Banyu Life Science Foundation International grant (Y.T).

Disclosures: A.Y. is an employee of Japan Bio Products Co., Ltd.

moderate, and severe stages) and proliferative DR (PDR; advanced stage) with neovascularization, preretinal or vitreous hemorrhage, and fibrovascular proliferation.¹ Usually, operative or laser treatment is chosen for advanced PDR, whereas NPDR and early stages of PDR are adequately treated with drugs.

Although the fundamental molecular pathogenesis of DR progression is still under discussion, evidence suggests that vascular endothelial growth factor (VEGF) is a key player in the onset and progression of DR and its complications.² This is because VEGF promotes hyperpermeability and inflammation of the retinal vasculature and neovascularization. In recent years, intravitreal administration of anti-VEGF agents has been used to treat DR,³ exudative age-related macular degeneration,⁴ retinal vein occlusion,⁵ and retinopathy of prematurity.⁶ However, this approach has several associated drawbacks. One is recurrence of macular edema; another is tachyphylaxis, which can occur after long-term anti-VEGF administration.^{7,8} Therefore, identification of other therapeutic targets involved in controlling vascular integrity would be desirable.

Originally isolated from human pheochromocytoma,⁹ adrenomedullin (ADM) is a vasodilating polypeptide whose expression is up-regulated under ischemic conditions.^{10–12} We previously showed that ADM knockout (*Adm*^{−/−}) mice die *in utero* because of vascular structural abnormalities,¹³ which is indicative of ADM's indispensability for proper vascular development. However, ADM exerts its vascular effects not only during development but also in adults.^{13,14} Moreover, ADM is now known to be widely distributed in numerous tissues and organs and to exert a variety of physiological effects in addition to vasodilatation. For example, ADM also acts as an antioxidant, anti-inflammatory, anti-fibrotic, and antiapoptotic mediator.^{15–17}

Increased plasma ADM levels are observed in diabetes,¹⁸ and several clinical studies have shown that there is an association between plasma ADM levels and DR. In type 1 diabetes, patients with DR show higher plasma ADM levels than patients without complications.¹⁹ Likewise, plasma ADM levels are elevated in patients with type 2 diabetes and DR.^{20–22} Vitreous ADM levels are also elevated in patients with DR,^{23–27} and plasma or vitreous ADM levels are reportedly related to the severity and stability of DR.^{20,26} These clinical data strongly implicate ADM in the pathogenesis of DR, although the specific function of ADM in DR remains to be clarified.^{28–30}

There is currently no standard animal model of spontaneous diabetes in which the phenotypic characteristics exactly mimic the pathogenesis of human DR. This is mostly because of the complexity of the pathologic changes. Nonetheless, several studies have reported using the C57BL/6-Ins2^{Akita}/J (Akita) mouse, which is a genetic model of type 1 diabetes,³¹ or the trVEGF029 (Kimba) mouse,³² which is a homozygous transgenic mouse line exhibiting photoreceptor (rhodopsin)-specific overexpression of human VEGF-165 (hVEGF165). More

recently, Akimba mice (Ins2^{Akita} VEGF^{+/-}) were generated from Kimba (VEGF^{+/-}) and Akita mice.³³ Akimba mice show retinal neovascularization and display advanced clinical PDR, including diffuse vascular leakage, edema, proliferative changes, and retinal detachment. In this study, we primarily used Kimba mice, which exhibit early and mild DR, for the purpose of assessing the potential of ADM to serve as an effective treatment for DR.

Materials and Methods

Animals

We used normal male wild-type (WT) C57BL/6J mice, male heterozygous Ins2^{Akita} mice on a C57BL/6J background (C57BL/6-Ins2^{Akita}/J; Akita), homozygous transgenic mice exhibiting photoreceptor (rhodopsin)-specific overexpression of hVEGF165 on a C57BL/6J background (trVEGF029; Kimba) and their age-matched WT littermates, and mice crossbred from Akita and Kimba (Ins2^{Akita} VEGF^{+/-}; Akimba; Lions Eye Institute, Nedlands, Australia).

Akita mice develop type 1 diabetes at around 4 weeks of age. Only male mice carrying a single Ins2^{Akita} allele are established as suitable models of diabetic complications. After genotyping, blood glucose levels were measured to ensure diabetic status. Genotyping for the Ins2^{Akita} allele was performed using PCR in a Veriti 96-Well Thermal Cycler (Thermo Fisher Scientific, Waltham, MA) with primers 5'-TGCTGATGCCCTGGCCTGCT-3' (forward) and 5'-TGGTCCCACATATGCACATG-3' (reverse), and with a restriction enzyme digestion protocol described by The Jackson Laboratory (Bar Harbor, ME) in protocol Ins2^{Akita}.

Kimba mice are a model of VEGF-induced retinopathy and exhibit hVEGF overexpression driven by a truncated mouse rhodopsin promoter. The continuous hVEGF overexpression drives retinal damage with phenotypic similarities to human NPDR and PDR, including areas of retinal vascular leakage, microaneurysms, moderate-to-severe capillary nonperfusion, and vascular proliferation. Genotyping for the hVEGF165 was performed using PCR with primers 5'-CGAGGCTCAGAGAGGAATACTT-3' (forward) and 5'-CAGCGCCTCGGCTTGTCAC-3' (reverse).³²

All animal handling procedures were in accordance with a protocol approved by the Ethics Committee of Shinshu University School of Medicine. All experiments were performed in accordance with the Association for the Research in Vision and Ophthalmology's Statement for the Use of Animals in Ophthalmic and Vision Research and the institutional guidelines of Shinshu University.

TEFI

Topical endoscopy fundus imaging (TEFI) was performed as described previously³⁴ with some modifications. We used an endoscope coupled to a 5-cm-long otoscope with a 3-mm outer diameter (1218AA; Karl Storz, Tuttlingen,

Germany). A reflex digital camera with an 18-megapixel CMOS image sensor (EOS REBEL T4i; Canon, Tokyo, Japan) was connected to the endoscope through an adapter. Pupils were dilated with topical 0.5% phenylephrine and 0.5% tropicamide (Mydrin-P; Santen, Osaka, Japan), which were applied 60 minutes and 30 minutes before eye examination, respectively. Mice were anesthetized with an intraperitoneal injection of tribromoethanol (Avertin; Wako, Osaka, Japan) just before the examination, after which their whiskers were shaved, and one drop of 0.4% oxybuprocaine was applied to each eye. The camera was placed on a platform, and the endoscope was slowly moved toward the mouse. Once the endoscope was in contact with the gel covering the cornea, the photographer adjusted its position by horizontally displacing the tip. Focus and illumination were adjusted during examination of the fundus through the camera.

Fluorescence Angiography

The cornea was kept moist using saline. Mice were manually held in front of a Heidelberg Retina Angiograph 2 confocal scanning-laser ophthalmoscope (Heidelberg Engineering GmbH, Heidelberg, Germany). Angiography was performed after intraperitoneal injection of 12 $\mu\text{L/g}$ 2.5% fluorescein sodium (Alcon, Freiburg, Germany). Images were taken at late phase (2–3 minutes) of fluorescein perfusion relative to the time of injection and used for quantitative evaluations. Vascular density, vascular area, and vascular branching points were quantified from the angiography images using the WimRetina image analysis system (Onimagin Technologies SCA, Córdoba, Spain).

Transmission Electron Microscopy

Specimens were fixed in 2.5% glutaraldehyde (pH 7.2), embedded in epoxy resin (Epok) 812 (Oken Shoji, Tokyo, Japan), cut into ultrathin sections, stained with uranyl acetate and lead citrate, and examined under an electron microscope. Experiments were repeated three times.

Lectin Staining of Retinas

Retinas were isolated and stained overnight at 4°C with Alexa Fluor 594–conjugated Griffonia bandeiraea simplicifolia isolectin B4 (Thermo Fisher Scientific) in phosphate-buffered saline (PBS) with 0.3% Triton X-100. After washing three times in PBS, the retinas were whole-mounted onto microscope slides with the photoreceptor side down and then embedded in fluorescence mounting medium (Agilent Technologies, Santa Clara, CA). Images of whole-mount retinas were taken at $\times 40$ magnification using a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan). Vascular density, vascular area, and vascular

branching points were quantified from lectin-stained images using the WimRetina image analysis system.

Evans Blue Permeability Assay

Evans Blue permeability assay was performed according to a previous study.³⁵ Evans Blue dye (30 mg/mL in PBS, 45 mg/kg; Sigma-Aldrich, St. Louis, MO) was injected through the tail vein. After 2 hours, 200 μL of blood was taken and assayed. Each mouse was perfused with 1% paraformaldehyde in 50 mmol/L sodium citrate, pH 3.5, at 37°C to clear Evans Blue in the vasculature. The eyes were enucleated, retinas were dissected, and dry weight was calculated. The Evans Blue dye was extracted with 100 μL of formamide, followed by ultracentrifugation. To assess the Evans Blue-albumin concentration, the absorbance of the retinal extract and plasma samples was measured at 620 nm and 740 nm. The retinal vascular permeability was calculated as the quantity of retinal Evans Blue normalized to retinal dry weight, plasma Evans Blue concentration, and circulation time by using the formula previously described.^{36,37}

In Vivo Leukocyte Transmigration Assay

In vivo leukocyte transmigration assays were performed as described previously³⁸ with some modifications. The animals were anesthetized and intraperitoneally injected with 10 $\mu\text{L/g}$ acridine orange (AO) (1 mg/mL; A6014; Sigma-Aldrich). Thirty minutes after injection, the AO concentrations within intravascular leukocytes and endothelial cells were significantly diminished because of a washout effect. By contrast, transmigrated leukocytes retained their staining, which enabled their visualization. Two hours after AO injection, AO-positive transmigrated leukocytes (outside the vessels) were observed using scanning-laser ophthalmoscope with a blue excitation light (488 nm).

Measurement of sORP

Mice were anesthetized, after which time the aqueous humor was collected using a 30-gauge needle and pooled into a siliconized microcentrifuge tube. Samples were then centrifuged at 3000 rpm for 3 minutes, and the cell-free supernatant fluid was immediately frozen at -80°C . On average, 5 μL of aqueous humor was obtained from the two eyes of each mouse. Static oxidation-reduction potential (sORP) was determined using a RedoxSYS Diagnostic System (Luoxis Diagnostics, Inc., Englewood, CO).³⁹ Twenty microliters of sample (5 μL of aqueous humor + 15 μL of dH_2O) were applied to disposable sensors, and sORP was measured for 4 minutes. sORP captures the integrated balance of oxidants and reductants in a specimen and expresses it in terms of mV.

Table 1 Primers Used for Real-Time PCR

Primer name	Sequence
h VEGF-165 forward	5'-CTACCTCCACCATGCCAAGT-3'
h VEGF-165 reverse	5'-GCAGTAGCTGCGTGATAGA-3'
m VEGF-A forward	5'-CATCTTCAAGCCGTCCTGTGT-3'
m VEGF-A reverse	5'-CTCCAGGGCTTCATCGTTACA-3'
m MCP-1 forward	5'-GCAGTTAACGCCCCACTCA-3'
m MCP-1 reverse	5'-CCTACTCATTTGGGATCATCTTGCT-3'
m ICAM-1 forward	5'-CCTAAAATGACCTGCAGACGG-3'
m ICAM-1 reverse	5'-TTTGACAGACTTCACCACCCC-3'
m VCAM-1 forward	5'-CCCTGAATACAAAACGATCGC-3'
m VCAM-1 reverse	5'-CAGCCCGTAGTGCTGCAAG-3'
m TNF- α forward	5'-ACGGCATGGATCTCAAAGAC-3'
m TNF- α reverse	5'-AGATAGCAAATCGGCTGACG-3'
m IL-1 β forward	5'-CTACAGGCTCCGAGATGAACAAC-3'
m IL-1 β reverse	5'-TCCATTGAGGTGGAGAGCTTTC-3'
m IL-6 forward	5'-CCCAATTTCCAATGCTCTCC-3'
m IL-6 reverse	5'-TGAATTGGATGGTCTTGGTCC-3'
m p22 phox forward	5'-GGCCATTGCCAGTGTGATCT-3'
m p22 phox reverse	5'-GCTCAATGGGAGTCCACTGC-3'
m p47 phox forward	5'-ATCCTATCTGGAGCCCCTTGA-3'
m p47 phox reverse	5'-CACCTGCGTAGTTGGGATCC-3'
m p67 phox forward	5'-CAGACCCAAACCCCAAGAA-3'
m p67 phox reverse	5'-AAAGCCAAACAATACGCGGT-3'
m eNOS forward	5'-AGGCACTGCTGAGCCGAGT-3'
m eNOS reverse	5'-TTCTCCAGTTGTTCCACAGCC-3'

h, human; m, mouse.

Quantitative Real-Time RT-PCR Analysis

Total RNA was isolated using a PureLink RNA Mini Kit (Thermo Fisher Scientific). RNA quality was verified using electrophoresis, and concentrations were measured using an Oubit 3.0 Fluorometer (Thermo Fisher Scientific). Thereafter, the extracted RNA was treated with DNA-Free (Thermo Fisher Scientific) to remove contaminating DNA, and 2- μ g samples were subjected to reverse transcription using a PrimeScript RT reagent Kit (Takara Bio, Shiga, Japan). Quantitative real-time RT-PCR was performed using a StepOne Plus Real-Time PCR System (Thermo Fisher Scientific) with SYBR green (Toyobo, Osaka, Japan) or Realtime PCR Master Mix (Toyobo) and TaqMan probe (MBL, Nagoya, Japan). Values were normalized to mouse GAPDH (Pre-Developed TaqMan assay reagents; Thermo Fisher Scientific). The primers used are listed in Table 1.

Real-Time RT-PCR Array Analysis

We used a PCR array (RT² Profiler PCR Array; Qiagen, Hilden, Germany) to assess expression of a focused panel of genes. One microgram of total RNA was converted to cDNA using a RT² First Strand Kit (Qiagen). The levels of specific transcripts were assessed using a Mouse NF- κ B Signaling Pathway PCR Array (Qiagen) according to the manufacturer's protocols. All PCR reactions were run using a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). RT² Profiler PCR Array data were analyzed

using RT² Profiler Array Data Analysis Software version 3.5 (Qiagen).

Western Blot Analysis

Retinal tissue was lysed in an ice-cold RIPA Lysis Buffer System (Santa Cruz Biotechnology, Santa Cruz, CA) supplemented with PosSTOP phosphatase inhibitor (Roche Applied Science, Upper Bavaria, Germany) and then sonicated. Samples of the resultant lysate were subjected to SDS-PAGE, and the resolved proteins were transferred to cellulose nitrate membranes (GE Healthcare, Little Chalfont, UK). After blocking in 5% skim milk, the membranes were incubated with primary antibodies against β -tubulin (H-235:sc-9104; Santa Cruz Biotechnology), VEGF-A (ab46154; Abcam, Cambridge, UK), intercellular adhesion molecule (ICAM)-1 (M-19:sc-1511; Santa Cruz Biotechnology), and vascular cell adhesion molecule (VCAM)-1 (C19:sc-1504; Santa Cruz Biotechnology), followed by appropriate secondary antibodies (Santa Cruz Biotechnology). The primary antibodies were used in 1:2000 dilution, and the secondary antibodies were used in 1:4000 dilution. The bound antibodies were visualized using a chemiluminescent horseradish peroxidase substrate (Merck Millipore, Darmstadt, German), and the chemiluminescence was analyzed using an Image Quant LAS 4000 system (GE Healthcare). For quantification, images of Western blot analysis were captured and analyzed using Scion Image software version 4.0.3.2.

Systemic Administration of ADM to Mice

Human ADM (Peptide Institute, Inc., Osaka, Japan) dissolved in PBS was infused into subcutaneous tissues using osmotic pumps (Alzet; Durect Co., Cupertino, CA). The delivery rate was 26 μ g/kg per day, and the mice received ADM for 7 days. Mice treated with PBS served as controls.

Intravitreal Administration of ADM to Mice

Human ADM dissolved in PBS (10^{-7} mol/L; 1.5 μ L) was intravitreally injected at the temporal corneoscleral junction using a Hamilton syringe equipped with a 32-gauge needle under a stereoscopic surgical microscope. As a control, eyes were intravitreally injected with 1.5 μ L of PBS. After the procedure, the mice were administered moxifloxacin hydrochloride antibiotic drops (Vigamox Ophthalmic Solution; Alcon, Fudenberg, Switzerland).

Endothelial Cells

TR-iBRB cell (FACT, Sendai, Japan), a conditionally immortalized rat retinal capillary endothelial cell line generated from rats harboring temperature-sensitive SV40 antigen, were used for this study. TR-iBRB cells were cultivated in Dulbecco's modified Eagle's medium

supplemented with 10% fetal bovine serum and antibiotics. Human umbilical vein endothelial cells (HUVECs; Lonza, Basel, Switzerland) were cultured in endothelial cell growth medium-2 (Lonza).

Transendothelial FITC Permeability

Permeability across endothelial cell monolayers was measured using gelatin-coated 24-well Falcon Cell Culture Plate and Inserts (Thermo Fisher Scientific). TR-iBRB cells were plated at a density of 1.0×10^5 cells per well (upper compartment) and then cultured for 3 days, by which time they formed a tight monolayer. Endothelial permeability was determined by measuring the passage of fluorescein isothiocyanate (FITC)-labeled dextran (1 mg/mL FITC-dextran; molecular mass, 70 kDa; Sigma-Aldrich) through the TR-iBRB monolayer. Thereafter, 10^{-7} mol/L ADM was added to the incubation medium (upper compartment), and after pretreatment for 1 hour, 20 ng/mL VEGF-A was added to the upper compartment in the presence of 1 mg/mL FITC-labeled dextran. After incubation for an additional 30 and 60 minutes, 100 μ L of medium was collected from the lower compartment, and fluorescence was evaluated using a Microplate fluorometer (SpectraMax 190 microplate reader; Molecular Devices, Sunnyvale, CA). An endothelial cell monolayer permeability index was calculated as described previously.⁴⁰

TEER

For transendothelial electrical resistance (TEER), 24-well Millicell Hanging Cell Culture Plate and Inserts 1.0 μ m polyethylene terephthalate (Merck Millipore) was used. TR-iBRB cells were plated at a density of 1.0×10^5 cells per well. TEER was measured in confluent TR-iBRB cells using a Millicell-ERS2 Epithelial Volt/Ohm Meter system (Merck Millipore) as described previously.⁴¹ After pretreatment with PBS or 10^{-8} to 10^{-6} mol/L ADM for 3 hours, 20 ng/mL VEGF was added to the cells, and this time point was defined as 0 minute. TEER was then measured at 0, 60, 120, and 180 minutes. Because the measured TEER included the resistance of the interelectrode solution and blank membrane, a transwell polycarbonate membrane culture dish without cells was always included as a blank. Every test was performed in triplicate and repeated at least three times.

Immunofluorescence Staining of Cultured Cells

TR-iBRB cells or HUVECs in slide chambers were washed with PBS and fixed in acetone and 4% paraformaldehyde for 15 minutes and rinsed in PBS. Cells were then treated with 1% bovine serum albumin at 37°C for 60 minutes. After washing the cells three times for 3 minutes each with PBS, they were incubated with anti-NF- κ B p65 (1:100 dilution; Abcam) or anti-ZO-1 (1:100 dilution; BD Biosciences,

Franklin Lakes, NJ) antibody in PBS containing 1% bovine serum albumin at 4°C overnight. Slides were then washed and incubated with a secondary antibody (1:200 dilution) in PBS containing 1% bovine serum albumin for 60 minutes at 4°C. Finally, the slides were washed, covered with Mounting Medium (Agilent Technologies), and observed under a fluorescence microscope.

Figure 1 Evaluation of the severity of retinopathy using topical endoscopy fundus imaging (TEFI). **A:** Ocular fundus images of WT, Akita, Kimba, and Akimba mice obtained using TEFI. Fundus images were classified as follows: 0, normal; I, NPDR; II, early PDR; III, advanced PDR. **B:** Distributions showing the changes in each mouse type revealed by TEFI. FA, fluorescent angiography; NPDR, nonproliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy; WT, wild-type.

NF- κ B p65 and I κ B α Detection

Whole-cell extracts were prepared from 1×10^3 TR-iBRB cells cultured with or without 20 ng/mL VEGF or 10^{-7} mol/L ADM for 3 hours at 37°C using a RIPA lysis buffer kit (Santa Cruz Biotechnology). NF- κ B p65 and I κ B α were analyzed using an InstantOne enzyme-linked immunosorbent assay (Affymetrix, Santa Cruz, CA), which uses a tetramethylbenzidine colorimetric substrate to detect total and phosphorylated (phospho)-NF- κ B p65 and phospho-I κ B α attached to consensus binding sites in a 96-well plate. Measurements were made based on the OD450, using a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA).

Statistical Analysis

Values are expressed as means $\pm$ SEM. The *t*-test and one-way analysis of variance followed by the Tukey's test were used to determine the significance of differences. Values of $*P < 0.05$ were considered significant.

Results

Evaluation of the Severity of Retinopathy in Akita, Kimba, and Akimba Mice

To visualize the ocular fundus of living mice, we applied TEFI to 8- to 10-week-old male mice as described previously (Figure 1A).³⁴ From the TEFI, each mouse's retinopathy was graded as follows: 0, normal; I, NPDR; II, early PDR; and III, advanced PDR. Grades I to III were all detected among Kimba mice (Figure 1B). Most Akimba mice showed proliferative changes and were classified as grade II or grade III. WT and most of the Akita mice showed no pathologic changes.

To evaluate retinal vascular changes, we next performed fluorescein angiography (FA) using confocal scanning-laser ophthalmoscope (Figure 2A). Depending on the severity of DR, various pathologic changes were observed. At the NPDR stage, capillary dropout and aneurysmal formation were detected, whereas at the PDR stage proliferative changes and vitreous hemorrhage were detected. We graded the severity of the vascular changes detected with FA as follows: 0, normal; I, capillary dropout without vascular leakage; II, appearance of aneurysm formations and slight vascular leakage; III, apparent vascular leakage; IV, severe vascular leakage; V, massive vascular leakage and proliferative changes; and VI, vitreous hemorrhage and retinal detachment. Kimba mice mainly ranged from grade I to IV (Figure 2B). About half of the Akimba mice showed PDR and were scored as grade V or VI. All of the WT and most of the Akita mice showed no apparent vascular changes and were classified into grade 0. From these observations, we selected Kimba mice as an adequate

Figure 2 Evaluation of the severity of retinopathy using fluorescein angiography (FA). **A:** FA showing the severity of retinopathy. The progression of diabetic retinopathy (DR) was divided as follows: 0, normal; I, capillary dropout without vascular leakage; II, appearance of aneurysmal formations and slight vascular leakage; III, apparent vascular leakage; IV, severe vascular leakage; V, massive vascular leakage and proliferative changes; VI, vitreous hemorrhage and retinal detachment. **B:** Distributions showing the changes in each mouse type revealed by FA. WT, wild-type.

experimental model, because most showed NPDR and early stages of PDR without vitreous hemorrhage or retinal detachment, which suggested they would be appropriate targets for therapy using drugs.

Figure 3 Evaluation of capillary dropout and vascular leakage in Kimba mice. **A:** Representative images of fluorescein angiography (FA) in wild-type (WT) and Kimba mice. **B:** Comparison of vascular density, vascular area, and numbers of vascular branching points per field quantified from FA images using the WimRetina image analysis system (Onimagin Technologies SCA, Córdoba, Spain). **C:** Representative flat-mounted lectin-stained retinal specimens from WT and Kimba mice. **D:** Vascular density, vascular area, and numbers of vascular branching points per field quantified from lectin-staining images. **E:** Comparison of retinal Evans Blue leakage ($\mu\text{L plasma} \times \text{g retina wet wt}^{-1} \cdot \text{h}^{-1}$) evaluated 2 hours after intravenous injection of Evans Blue. **F:** Electron micrographs showing retinal vascular pathologic process. Superficial retinal vessels neighboring the vitreous body are shown. **Arrows** indicate the partial fragmentation of the vascular wall in Kimba mice. Data are expressed as means $\pm$ SEM. $n = 5$ in each group (**B**); $n = 4$ in each group (**D** and **E**). * $P < 0.05$, ** $P < 0.01$ (t -test). Scale bars = $2 \mu\text{m}$ (**F**).

Retinas of Kimba Mice Exhibit the Early Stages of DR

In the early stages of DR, capillary dropout, which is caused by obliteration of retinal capillaries, is the most commonly observed vascular change. To quantify capillary dropout in 8- to 10-week-old WT and Kimba mice, we performed FA and then stained the retinas with isolectin. With the use of the grading score in Figure 2B, we selected mice exhibiting grade I to IV changes, without proliferative changes. Then using the WimRetina image analysis system, we quantified the vascular density, vascular area, and the branching points of retinal vessels. FA revealed that Kimba mice experienced significant vascular loss because of capillary dropout (Figure 3, A and B), which was confirmed by direct vascular visualization of flat-mounted lectin-stained retinas (Figure 3, C and D). Also detected within the lectin-stained retinas of Kimba mice (Figure 3C) were aneurysmal formation and abnormal neovascularization, which are also characteristic

features of DR. These changes appeared to be a response to vascular loss and hypoxia, although they could not compensate for the total vascular loss in the retinas of Kimba mice.

Increased vascular permeability is another feature of early DR. To evaluate retinal vessel permeability *in vivo*, we performed Evans Blue assays as described previously.³⁷ We found that the vascular permeability of Kimba mice were about five times that of WT mice (Figure 3E). Consistent with this finding, electron microscopic observation revealed a fragile vascular structure with partially fragmented vascular walls in Kimba mice (Figure 3F).

Inflammation and Oxidative Stress Are Augmented in the Retinas of Kimba Mice

Because vascular permeability is strongly affected by inflammatory responses, we next used quantitative real-time

Figure 4 Increased inflammation in Akita and Kimba mice. **A** and **B**: Real-time PCR analysis of inflammation-related molecules in the retinal tissues from 9- to 10-week-old male Akita (**A**) and Kimba (**B**) mice compared with wild-type (WT) mice. Mean of the WT group was assigned a value of 1. **C**: Western blot analysis showing the expression levels of VEGF-A, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1) in the retinas of Kimba and WT mice. Experiments were repeated three times. **D** and **E**: *In vivo* leukocyte transmigration assay. **D**: Two hours after intraperitoneal injection of acridine orange (AO), AO-positive extravascular leukocytes (shown in red circles) were observed using scanning-laser ophthalmoscopy (SLO). **E**: Comparison of the numbers of transmigrated leukocytes/field between WT and Kimba mice. Data are expressed as means $\pm$ SEM. $n = 4$ mice in each group; $n = 2$ retinas from each mouse. * $P < 0.05$, ** $P < 0.01$ (t -test). hVEGF165, human vascular growth factor-165; MCP, monocyte chemoattractant protein; PBS, phosphate-buffered saline; TNF, tumor necrosis factor; WT, wild-type.

PCR to assess the retinal expression of several inflammation-related genes. In particular, MCP-1, VCAM-1, ICAM-1, IL-6, and TNF- α are reportedly important for the pathogenesis of diabetic macular edema (DME).² In 9- to 10-week-old Akita mice, all of these genes, except for VCAM-1, were

significantly elevated compared with their WT littermates (Figure 4A), which demonstrates that the high-glucose status in Akita mice can evoke retinal inflammation. This also suggested that subclinical retinal inflammation may already be present in the Akita mice studied, even

Figure 5 Increased oxidative stress in Akita and Kimba mice. **A** and **B**: Quantitative real-time PCR analysis of NADPH-oxidase subunits and eNOS in the retinal tissues from 9- to 10-week-old male Akita (**A**) and Kimba (**B**) mice compared with WT mice. Mean of the WT group was assigned a value of 1. **C**: Quantification of the static oxidation-reduction potential (sORP) in the samples of aqueous humor. sORP is measured in millivolts (mV). Data are expressed as means $\pm$ SEM. $n = 4$ mice in each group; $n = 2$ retinas from each mouse. * $P < 0.05$, ** $P < 0.01$ (t -test). eNOS, endothelial nitric oxide synthase; WT, wild-type.

though no apparent vascular leakage was observed (Figure 2A).

In addition to the inflammation-related genes, VEGF-A expression was also significantly elevated in Akita mice. In Kimba mice, elevation of the aforementioned inflammation-related genes was even more pronounced than in Akita mice (Figure 4B) as were levels of their proteins (Figure 4C). In particular, MCP-1 and tumor necrosis factor (TNF)- α expression was about 10 times higher in Kimba mice than in WT mice. Overall, the expression pattern of inflammatory molecules in Kimba mice was related to their retinal over-expression of VEGF-A and closely resembled the pattern in Akita mice.

Leukocyte adhesion to endothelium and transmigration into the vascular wall are key pathophysiological events during the onset of vascular inflammation.⁴² We therefore performed *in vivo* leukocyte transmigration assays (Figure 4D). Intraperitoneally injected AO is efficiently taken up by blood cells, after which the AO concentration in intravascular blood cells diminishes soon because of a washout effect. By contrast, extravasated and transmigrated leukocytes retain their staining, which allows their visualization. In Kimba mice, numbers of transmigrated leukocytes were significantly higher than in WT mice (Figure 4E).

Inflammation increases oxidative stress and vice versa, and both coordinately act within a vicious cycle to promote vascular lesions. Oxidative stress represented by increased expression of NADPH-oxidase subunits was significantly elevated in both Akita (Figure 5A) and Kimba mice (Figure 5B). In Kimba mice, endothelial nitric oxide synthase expression was also significantly elevated, probably as a compensatory response to the elevated oxidative stress and ischemia. In addition, the sORP measured in samples of aqueous humor, which generally reflect the oxidative stress level in the whole eye, was higher in both Akita and Kimba mice than in WT mice (Figure 5C).

ADM Suppresses Vascular Leakage and Vascular Loss in Kimba Mice

From the observations summarized above, we selected Kimba mice to evaluate the effects of ADM treatment on retinopathy. We initially continuously administered ADM or PBS systemically to WT and Kimba mice for 1 week using a subcutaneously implanted osmotic pump. FA was performed before (6- to 7-week-old mice) and after (8- to 9-week-old mice) the administration, and vascular leakage was evaluated using the leakage grading score

Figure 6 Systemic or intravitreal administration of adrenomedullin (ADM) suppresses vascular leakage in the retinas of Kimba mice. **A** and **B**: Representative images of fluorescent angiography (FA) in wild-type (WT) (**A**) and Kimba (**B**) mice at 6 to 7 and 8 to 9 weeks of age. Phosphate-buffered saline (PBS) or ADM was systemically administered for 1 week using an osmotic pump, and progression of vascular leakage during the observation period was recorded in the same mice. **C**: Comparison of the vascular leakage grade between 6- to 7- and 8- to 9-week-old Kimba mice treated with PBS or ADM. **D** and **E**: Representative images of FA in WT (**D**) and Kimba (**E**) mice treated with intravitreal administration of PBS or ADM. Images were acquired 48 hours after treatment with FA. **F**: Comparison of vascular leakage grades before and after treatment with PBS or ADM in Kimba mice. The progression of DR was divided as follows: 0, normal; I, capillary dropout without vascular leakage; II, appearance of aneurysmal formations and slight vascular leakage; III, apparent vascular leakage; IV, severe vascular leakage; V, massive vascular leakage and proliferative changes; VI, vitreous hemorrhage and retinal detachment. Data are expressed as means $\pm$ SEM. $n = 7$ in each group (**C**). $n = 5$ in each group (**F**). * $P < 0.05$ (t -test). W, week.

shown in Figure 2B. In WT mice (Figure 6A), ADM administration caused capillary dilatation, whereas PBS had no apparent effect. Kimba mice exhibited spontaneous progression of their retinopathy during the administration period. In Kimba mice receiving PBS, progression of the vascular leakage was observed (Figure 6, B and C). By contrast, in ADM-treated Kimba mice, progression of the vascular leakage was suppressed during the observation period (Figure 6, B and C). Similar results were obtained after intravitreal administration of ADM to 6- to 7-week-old WT (Figure 6D) and Kimba

(Figure 6E) mice. After 48 hours, ADM-treated Kimba mice showed a tendency toward suppression of vascular leakage (Figure 6, E and F).

In flat-mounted lectin-stained retinal specimens from ADM-treated Kimba mice, both capillary dropout and aneurysmal formation were suppressed (Figure 7A), as reflected by vascular density, vascular area, and vascular branching points within the whole retinas (Figure 7B). These observations clearly showed that in Kimba mice, ADM suppresses both vascular leakage and vessel loss, which are characteristic features of DR.

Figure 7 Adrenomedullin (ADM) administration suppresses capillary dropout and aneurysmal formation in the retinas of Kimba mice. **A:** Representative flat-mounted lectin-stained retinal specimens from Kimba mice. Kimba mice were systemically treated with phosphate-buffered saline (PBS) or ADM for 1 week. **B:** Vascular density, vascular area, and numbers of vascular branching points in whole retinas from Kimba mice were quantified from lectin-stained images using the WimRetina image analysis system (Onimagin Technologies SCA, Córdoba, Spain). Data are expressed as means $\pm$ SEM. $n = 3$ in each group. * $P < 0.05$, *** $P < 0.001$ (t -test).

ADM Suppresses VEGF-Evoked Hyperpermeability of Endothelial Cell Monolayers *in Vitro*

From the observed effects of ADM, we speculated that ADM could suppress VEGF-evoked retinal vascular

hyperpermeability. To test that hypothesis, we analyzed transendothelial permeability using TR-iBRB cells, an immortalized retinal capillary endothelial cell line. Endothelial permeability was determined by measuring the passage of FITC-labeled dextran from the upper to the

Figure 8 Adrenomedullin (ADM) suppresses VEGF-evoked endothelial cell hyperpermeability. **A:** Effects of 20 ng/mL VEGF and/or 10^{-7} mol/L ADM on the fluorescein isothiocyanate (FITC)-dextran permeability of endothelial cell monolayers. TR-iBRB cells were cultured on a semipermeable membrane until they formed a tight monolayer. Endothelial permeability was determined by measuring the passage of FITC-labeled dextran through the TR-iBRB monolayer. The endothelial cell monolayer permeability index was expressed in terms of 10^{-6} cm/s. **B:** trans-endothelial electrical resistance (TEER) of endothelial cell monolayers. The time course of TEER in the TR-iBRB cell monolayer was measured using a Millicell-ERS2 Epithelial Volt/Ohm Meter system. Cells were stimulated with 20 ng/mL VEGF with or without 10^{-8} to 10^{-6} mol/L ADM. TEER measured at the start of the stimulation was defined as 100%. Every test was performed in triplicate and repeated at least three times, and similar results were obtained each time. **C:** Fluorescent immunostaining of a tight junction molecule, ZO-1 (green) and DAPI (blue) in the human umbilical vein endothelial cell (HUVEC) monolayer. VEGF (20 ng/mL) treatment loosens and ADM (10^{-7} mol/L) treatment strengthens tight junction formation in the cultured cells. Data are expressed as means $\pm$ SEM. $n = 6$ in each group (**A** and **B**); $n = 3$ in each group (**C**). * $P < 0.05$ (one-way analysis of variance); * $P < 0.05$ phosphate-buffered saline (PBS) versus VEGF (one-way analysis of variance) (**B**). Scale bars = 40 μ m.

Figure 9 Adrenomedullin (ADM) suppresses VEGF-evoked endothelial cell inflammation. **A:** Quantitative real-time PCR analysis of inflammatory molecules in the cultured TR-iBRB cells. Cells were treated with phosphate-buffered saline (PBS), 20 ng/mL VEGF, or 20 ng/mL VEGF + 10^{-8} to 10^{-6} mol/L ADM. In the VEGF + ADM groups, the cells were pretreated first with ADM for 3 hours and then with VEGF for 3 hours. Mean of the PBS group was assigned a value of 1. **B:** Quantification using enzyme-linked immunosorbent assay (ELISA) of NF-κB p65 and phospho-NF-κB p65 (Ser 536), and IκBα and phospho-IκBα in whole-cell extract from TR-iBRB cells stimulated with PBS, 20 ng/mL VEGF, or 20 ng/mL VEGF + 10^{-7} mol/L ADM. In the VEGF + ADM groups, the cells were pretreated first with ADM for 3 hours and then with VEGF for 3 hours. Mean of the PBS group was assigned a value of 1. **C:** Fluorescent immunostaining of NF-κB p65 (green), phalloidin (red), and DAPI (blue) in the cultured TR-iBRB cells. Nuclear distribution of NF-κB p65 caused by VEGF is suppressed by co-administration of ADM. Data are expressed as means ± SEM. $n = 9$ MCP-1; $n = 7$ ICAM-1 and TNF-α. * $P < 0.05$ (one-way analysis of variance). Scale bars = 100 μm. ICAM, intercellular adhesion molecule; MCP, monocyte chemoattractant protein; TNF, tumor necrosis factor.

lower compartments of Transwell chambers through a monolayer of TR-iBRB cells cultured on the Transwell insert. Our findings indicated that VEGF significantly increased endothelial permeability and that co-administration of ADM suppressed the effect (Figure 8A). Consistent with that observation, VEGF also significantly reduced the TEER, and ADM inhibited the reduction of TEER elicited by VEGF (Figure 8B). ADM also strengthened tight junction formation in cultured HUVECs (Figure 8C). These data suggested that VEGF-induced disruption of endothelial barrier function was ameliorated by ADM treatment.

ADM Suppresses VEGF-Induced Inflammation

Stimulation of TR-iBRB cells with VEGF led to up-regulated expression of several inflammatory molecules, including MCP-1, ICAM-1, and TNF-α (Figure 9A). ADM dose dependently suppressed this inflammatory effect of VEGF-A. Because NF-κB orchestrated the

transcription of a wide range of inflammation-related molecules,⁴³ we evaluated NF-κB pathway activation using enzyme-linked immunosorbent assays to measure levels of phospho-NF-κB p65 and phospho-IκBα levels in whole cell extracts (Figure 9B) and by using fluorescent immunostaining to analyze the distribution of phospho-NF-κB p65 within nuclei (Figure 9C). We found that VEGF elevated NF-κB pathway activation but that co-administration of ADM suppressed this effect. In NF-κB proteome profiler array analysis, we also confirmed that VEGF administration on HUVECs significantly elevated levels of NF-κB p65, phospho-IκBα, and TNF receptor superfamily member 1A, 1B. However, co-administration of VEGF and ADM canceled it (Supplemental Figure S1).

To test whether ADM could suppress VEGF-evoked inflammation *in vivo*, we performed a comprehensive gene expression analysis using a real-time PCR array for NF-κB-related molecules in the retinas of Kimba mice, with or without ADM treatment. Nearly all NF-κB-related

A

Figure 10 Adrenomedullin (ADM) suppresses NF- κ B-related molecules in the retina of Kimba mice. **A:** Color panels showing the result of a real-time PCR array of NF- κ B-related molecules. Each column shows the result from one mouse. Red and green colors represent higher and lower gene expression, respectively. The retinas of Kimba mice show elevation of the indicated genes compared with wild-type (WT) mice. ADM was administered systemically for 1 week (week 7 to 8–week 9 to 10). **B:** Bar graphs showing comparison of representative inflammation-related genes in the real-time PCR array. Mean of the WT mice was assigned a value of 1. Data are expressed as means $\pm$ SEM. $n = 4$ in each group (**A**); $n = 3$ (**B**). * $P < 0.05$, ** $P < 0.01$ (one-way analysis of variance). ICAM, intercellular adhesion molecule. MCP, monocyte chemoattractant protein; VCAM, vascular cell adhesion molecule.

B

molecules were up-regulated in the retinas of Kimba mice compared with WT mice, and systemic administration of ADM suppressed their expression (Figure 10). In addition, in live imaging using AO, we observed that the rolling of leukocytes in the walls of arteries was increased after VEGF treatment and that effect too was suppressed by co-administration of ADM (Supplemental Figure S2). These data clearly showed that ADM suppressed VEGF-induced inflammation.

Discussion

Nearly 1 in 10 adults have now been diagnosed with diabetes,⁴⁴ and DR is a leading cause of blindness, estimated to affect between 28% and 40% of diabetic patients older than 40 years.⁴⁵ Microvascular sequelae are present in 25% of diabetic patients⁴⁶ and are the primary mechanism responsible for DR.⁴⁷ Although the pathologic changes associated with DR are complicated and the precise mechanisms

remain to be clarified, it seems clear that hyperglycemia is the cause of its onset.⁴⁸ Diabetic hyperglycemia disrupts retinal neurovascular units through biochemical and metabolic abnormalities that may damage endothelial cells or may induce apoptosis of endothelial cells, pericytes, microglia, and neurons.^{46,49,50} VEGF is now recognized to be a key player involved in DR. Intravitreal injection of VEGF is sufficient to produce many of the vascular abnormalities observed in DR.⁵¹ In addition to angiogenesis, VEGF promotes vascular permeability, and anti-VEGF agents have been used to suppress vascular hyperpermeability in DR. However, the effects of anti-VEGF agents on inflammation remain controversial. VEGF causes inflammation by inducing I κ B/NF- κ B,⁵² whereas anti-VEGF agents (ranibizumab and aflibercept) reportedly increase levels of the inflammatory adhesion molecules ICAM-1 and VCAM-1 on human endothelial cells.⁵³ Therefore, usage of anti-VEGF agents alone may stimulate inflammation, which may limit their use for chronic therapy.

We sought to affect retinal vascular integrity using therapeutic agents that do not target growth factors. ADM has anti-inflammatory actions that may be related to NF- κ B signaling.⁵⁴ ADM also reportedly inhibits VEGF-induced expression of inflammatory molecules through the phosphatidylinositol 3'-kinase–Akt pathway.⁵⁵ For those reasons, we speculated that ADM could be a novel candidate for DR therapy.

There are currently no standard animal models with phenotypic characteristics that exactly mimic the pathologic process of human DR. We therefore initially evaluated which animal model was most suitable for this study. Akita mice develop insulin-dependent diabetes, including hyperglycemia, and elevated vascular permeability is observed at later stages. However, few if any of the outstanding features of DR were observed in the younger mice that we used.⁵⁶ By contrast, Akimba mice showed advanced PDR, including diffuse vascular leakage, edema, proliferative changes, and retinal detachment.³³ Kimba mice reportedly exhibit pathologic changes to the retinal vasculature at 3 to 4 weeks of age, and stable retinopathy persists for 3 months.^{32,57,58} With the use of fundus imaging and angiography, we confirmed that most 8- to 10-week-old Kimba mice did not show retinal hemorrhage or detachment, but capillary dropout, aneurysmal formation, and vascular leakage, which resembled human NPDR and early-stage PDR, were noted. More interestingly, inflammation and oxidative stress were apparent in the retinas of Kimba mice. Retinal vascular leakage and inflammation are associated with retinal leukostasis in experimental diabetes,⁵⁹ and we confirmed enhanced leukocyte transmigration into the vascular wall in retinas from Kimba mice. From these observations, we deemed Kimba mice to be an adequate model with which to evaluate ADM treatment for retinopathy. It has also been reported that intravitreal injection of buffer may affect the progression of retinal diseases in mice,⁶⁰ and we found that intravitreal injection of PBS enhanced inflammation in the retinas of WT mice

(Supplemental Figure S3) but suppressed retinal inflammation in Kimba mice (Supplemental Figure S4). We therefore mainly administered ADM systemically in this study.

It was previously determined in clinical studies that plasma ADM levels are elevated in patients with DR.^{20–22} Moreover, vitreous ADM levels are also elevated in DR patients,^{23–27} and vitreous ADM is higher in DR patients with DME.²⁶ These clinical data suggest that ADM is produced locally in the eyes and is involved in the pathogenesis of DR, although the specific function of ADM in DR remains unclear.^{28–30} In the present study, we also found that ADM expression is elevated in the retinas of Kimba mice (data not shown). Given that ADM expression in endothelial cells is up-regulated by ischemia and inflammation,⁶¹ the elevation of ADM could be a compensatory response to DR. Consistent with that idea, real-time PCR array analysis revealed that systemic ADM administration suppressed retinal expression of inflammatory molecules in Kimba mice. To our knowledge, there have been no previous reports of molecules that could suppress such a wide range of inflammatory molecules within retinas. In the context of the controversy around the effects of anti-VEGF agents on inflammation, ADM could be an attractive alternative therapeutic agent.

The contributions made by ADM to endothelial cell growth and survival have been well reported.^{62–66} ADM enhances nitric oxide production and stimulates endothelial cell proliferation and migration; however, it suppresses apoptosis. We previously reported that ADM knockout mice (*Adm*^{−/−}) die *in utero* because of vascular structural abnormalities,¹³ which highlights the importance of ADM to proper vascular development. We also showed that the vascular function of ADM is mainly regulated by its receptor activity-modifying protein, receptor activity-modifying protein 2 (RAMP2), because *Ramp2*^{−/−} mice exhibited a

Figure 11 Actions and therapeutic potential of adrenomedullin (ADM) in diabetic retinopathy. ADM suppresses chronic inflammation and oxidative stress in diabetic retinopathy (DR), which is mainly attributed to chronic retinal VEGF–NF- κ B activation. ADM also suppresses vascular hyperpermeability. Chronic inflammation and vascular hyperpermeability coordinately promote and exacerbate DR. By breaking this vicious cycle, ADM could be an alternative to and/or adjuvant therapy with anti-VEGF agents for the treatment of retinopathy.

lethal phenotype nearly identical to that of *Adm*^{-/-} mice.⁶⁷ Likewise, the phenotypes of vascular endothelial cell-specific *Ramp2*^{-/-} (*E-Ramp2*^{-/-}) were embryonically lethal with systemic edema and vascular abnormalities, including malformation and partial detachment of endothelial cells, and enhanced vascular leakage. From these observations, we speculated that ADM could positively affect endothelial cell integrity within the retinal vasculature. Consistent with that idea, we observed that systemic or intravitreal ADM administration suppressed retinal vascular leakage in Kimba mice. In particular, systemic ADM administration significantly suppressed the spontaneous progression of vascular leakage otherwise observed in Kimba mice. With the use of retinal endothelial cells, we also showed that ADM suppresses VEGF-induced hyperpermeability. *In vitro* studies indicate that ADM improves the barrier function of endothelial cells, at least in part by strengthening tight junction formation. We therefore conclude that ADM plays a central role in retinal vascular integrity and that ADM treatment could potentially suppress both the inflammation and vascular hyperpermeability seen in DR.

A major cause of visual disturbance in DR is DME, which may occur at any stage of diabetes, even before a diagnosis of DR is made. Once DME develops, it often becomes chronic and intractable.⁴⁸ DME is caused by vascular leakage resulting from compromise of the blood-retinal barrier. As with other retinal diseases, DME is multifactorial,⁴⁸ although inflammation likely plays a substantial role. DME can be thought of as a state of chronic low-grade inflammation that accompanies vascular leakage.^{42,68} By suppressing inflammation and vascular leakage, ADM may suppress the BRB damage that leads to DME.

Figure 11 summarizes the actions and therapeutic potential of ADM in DR. ADM suppresses chronic inflammation and oxidative stress in DR, which is mainly attributed to chronic retinal VEGF–NF-κB activation. ADM also suppresses vascular hyperpermeability, which is the main cause of DME. Chronic inflammation and vascular hyperpermeability coordinately promote and exacerbate DR. By breaking this vicious cycle, ADM could be an alternative to and/or adjuvant therapy with anti-VEGF agents for the treatment of retinopathy.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2017.01.014>.

References

- Cheung N, Mitchell P, Wong TY: Diabetic retinopathy. *Lancet* 2010, 376:124–136
- Fogli S, Mogavero S, Egan CG, Del Re M, Danesi R: Pathophysiology and pharmacological targets of VEGF in diabetic macular edema. *Pharmacol Res* 2016, 103:149–157
- Nicholson BP, Schachat AP: A review of clinical trials of anti-VEGF agents for diabetic retinopathy. *Graefes Arch Clin Exp Ophthalmol* 2010, 248:915–930
- Wong TY, Liew G, Mitchell P: Clinical update: new treatments for age-related macular degeneration. *Lancet* 2007, 370:204–206
- Wong TY, Scott IU: Clinical practice. Retinal-vein occlusion. *N Engl J Med* 2010, 363:2135–2144
- Mintz-Hittner HA, Kennedy KA, Chuang AZ; BEAT-ROP Cooperative Group: Efficacy of intravitreal bevacizumab for stage 3+ retinopathy of prematurity. *N Engl J Med* 2011, 364:603–615
- Schaal S, Kaplan HJ, Tezel TH: Is there tachyphylaxis to intravitreal anti-vascular endothelial growth factor pharmacotherapy in age-related macular degeneration? *Ophthalmology* 2008, 115: 2199–2205
- Keane PA, Liakopoulos S, Ongchin SC, Heussen FM, Msuttha S, Chang KT, Walsh AC, Sadda SR: Quantitative subanalysis of optical coherence tomography after treatment with ranibizumab for neovascular age-related macular degeneration. *Invest Ophthalmol Vis Sci* 2008, 49:3115–3120
- Kitamura K, Kangawa K, Kawamoto M, Ichiki Y, Nakamura S, Matsuo H, Eto T: Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. *Biochem Biophys Res Commun* 1993, 192:553–560
- Fujita Y, Mimata H, Nasu N, Nomura T, Nomura Y, Nakagawa M: Involvement of adrenomedullin induced by hypoxia in angiogenesis in human renal cell carcinoma. *Int J Urol* 2002, 9:285–295
- Wang X, Yue TL, Barone FC, White RF, Clark RK, Willette RN, Sulpizio AC, Aiyar NV, Ruffolo RR Jr, Feuerstein GZ: Discovery of adrenomedullin in rat ischemic cortex and evidence for its role in exacerbating focal brain ischemic damage. *Proc Natl Acad Sci U S A* 1995, 92:11480–11484
- Nguyen SV, Claycomb WC: Hypoxia regulates the expression of the adrenomedullin and HIF-1 genes in cultured HL-1 cardiomyocytes. *Biochem Biophys Res Commun* 1999, 265:382–386
- Shindo T, Kurihara Y, Nishimatsu H, Moriyama N, Kakoki M, Wang Y, Imai Y, Ebihara A, Kuwaki T, Ju KH, Minamino N, Kangawa K, Ishikawa T, Fukuda M, Akimoto Y, Kawakami H, Imai T, Morita H, Yazaki Y, Nagai R, Hirata Y, Kurihara H: Vascular abnormalities and elevated blood pressure in mice lacking adrenomedullin gene. *Circulation* 2001, 104:1964–1971
- Iimuro S, Shindo T, Moriyama N, Amaki T, Niu P, Takeda N, Iwata H, Zhang Y, Ebihara A, Nagai R: Angiogenic effects of adrenomedullin in ischemia and tumor growth. *Circ Res* 2004, 95:415–423
- Brain SD, Grant AD: Vascular actions of calcitonin gene-related peptide and adrenomedullin. *Physiol Rev* 2004, 84:903–934
- Kato J, Tsuruda T, Kita T, Kitamura K, Eto T: Adrenomedullin: a protective factor for blood vessels. *Arterioscler Thromb Vasc Biol* 2005, 25:2480–2487
- Shimosawa T, Ogihara T, Matsui H, Asano T, Ando K, Fujita T: Deficiency of adrenomedullin induces insulin resistance by increasing oxidative stress. *Hypertension* 2003, 41:1080–1085
- Hayashi M, Shimosawa T, Isaka M, Yamada S, Fujita R, Fujita T: Plasma adrenomedullin in diabetes. *Lancet* 1997, 350:1449–1450
- Garcia-Unzueta MT, Montalban C, Pesquera C, Berrazueta JR, Amado JA: Plasma adrenomedullin levels in type 1 diabetes. Relationship with clinical parameters. *Diabetes Care* 1998, 21:999–1003
- Nakamura T, Honda K, Ishikawa S, Kitamura K, Eto T, Saito T: Plasma adrenomedullin levels in patients with non-insulin dependent diabetes mellitus: close relationships with diabetic complications. *Endocr J* 1998, 45:241–246
- Zakareia FA, Alderees AA, Al Regaiy KA, Alrouq FA: Correlation of electroretinography b-wave absolute latency, plasma levels of human basic fibroblast growth factor, vascular endothelial growth factor, soluble fatty acid synthase, and adrenomedullin in diabetic retinopathy. *J Diabetes Complications* 2010, 24:179–185
- Caliumi C, Balducci S, Petramala L, Cotesta D, Zinamosca L, Cianci R, Di Donato D, Vingolo EM, Fallucca F, Letizia C: Plasma

- levels of adrenomedullin, a vasoactive peptide, in type 2 diabetic patients with and without retinopathy. *Minerva Endocrinol* 2007, 32: 73–78
23. Ito S, Fujisawa K, Sakamoto T, Ishibashi T: Elevated adrenomedullin in the vitreous of patients with diabetic retinopathy. *Ophthalmologica* 2003, 217:53–57
 24. Udono T, Takahashi K, Abe T, Shibahara S, Tamai M: Elevated immunoreactive-adrenomedullin levels in the aqueous humor of patients with uveitis and vitreoretinal disorders. *Peptides* 2002, 23: 1865–1868
 25. Er H, Doganay S, Ozerol E, Yurekli M: Adrenomedullin and leptin levels in diabetic retinopathy and retinal diseases. *Ophthalmologica* 2005, 219:107–111
 26. Lu Y, Xu Y, Tang C: Changes in adrenomedullin in patients with proliferative diabetic retinopathy. *Curr Eye Res* 2011, 36:1047–1052
 27. Udono T, Takahashi K, Takano S, Shibahara S, Tamai M: Elevated adrenomedullin in the vitreous of patients with proliferative vitreoretinopathy. *Am J Ophthalmol* 1999, 128:765–767
 28. Iesato Y, Toriyama Y, Sakurai T, Kamiyoshi A, Ichikawa-Shindo Y, Kawate H, Yoshizawa T, Koyama T, Uetake R, Yang L, Yamauchi A, Tanaka M, Igarashi K, Murata T, Shindo T: Adrenomedullin-RAMP2 system is crucially involved in retinal angiogenesis. *Am J Pathol* 2013, 182:2380–2390
 29. Yuda K, Takahashi H, Inoue T, Ueta T, Iriyama A, Kadonosono K, Tamaki Y, Aburatani H, Nagai R, Yanagi Y: Adrenomedullin inhibits choroidal neovascularization via CCL2 in the retinal pigment epithelium. *Am J Pathol* 2012, 181:1464–1472
 30. Iesato Y, Yuda K, Chong KT, Tan X, Murata T, Shindo T, Yanagi Y: Adrenomedullin: a potential therapeutic target for retinchoroidal disease. *Prog Retin Eye Res* 2016, 52:112–129
 31. Han Z, Guo J, Conley SM, Naash MI: Retinal angiogenesis in the Ins2(Akita) mouse model of diabetic retinopathy. *Invest Ophthalmol Vis Sci* 2013, 54:574–584
 32. Lai CM, Dunlop SA, May LA, Gorbato M, Brankov M, Shen WY, Binz N, Lai YK, Graham CE, Barry CJ, Constable IJ, Beazley LD, Rakoczy EP: Generation of transgenic mice with mild and severe retinal neovascularisation. *Br J Ophthalmol* 2005, 89: 911–916
 33. Rakoczy EP, Ali Rahman IS, Binz N, Li CR, Vagaja NN, de Pinho M, Lai CM: Characterization of a mouse model of hyperglycemia and retinal neovascularization. *Am J Pathol* 2010, 177: 2659–2670
 34. Paques M, Guyomard JL, Simonutti M, Roux MJ, Picaud S, Legargasson JF, Sahel JA: Panretinal, high-resolution color photography of the mouse fundus. *Invest Ophthalmol Vis Sci* 2007, 48: 2769–2774
 35. Liu H, Ren JG, Cooper WL, Hawkins CE, Cowan MR, Tong PY: Identification of the antivasopermeability effect of pigment epithelium-derived factor and its active site. *Proc Natl Acad Sci U S A* 2004, 101:6605–6610
 36. Qaum T, Xu Q, Joussen AM, Clemens MW, Qin W, Miyamoto K, Hassessian H, Wiegand SJ, Rudge J, Yancopoulos GD, Adamis AP: VEGF-initiated blood-retinal barrier breakdown in early diabetes. *Invest Ophthalmol Vis Sci* 2001, 42:2408–2413
 37. Xu Q, Qaum T, Adamis AP: Sensitive blood-retinal barrier breakdown quantitation using Evans blue. *Invest Ophthalmol Vis Sci* 2001, 42:789–794
 38. Nakao S, Arita R, Nakama T, Yoshikawa H, Yoshida S, Enaida H, Hafezi-Moghadam A, Matsui T, Ishibashi T: Wide-field laser ophthalmoscopy for mice: a novel evaluation system for retinal/choroidal angiogenesis in mice. *Invest Ophthalmol Vis Sci* 2013, 54:5288–5293
 39. Stagos D, Goutzourelas N, Ntontou AM, Kafantaris I, Deli CK, Poullos A, Jamurtas AZ, Bar-Or D, Kouretas D: Assessment of eccentric exercise-induced oxidative stress using oxidation-reduction potential markers. *Oxid Med Cell Longev* 2015, 2015: 204615
 40. Kis B, Deli MA, Kobayashi H, Abraham CS, Yanagita T, Kaiya H, Isse T, Nishi R, Gotoh S, Kangawa K, Wada A, Greenwood J, Niwa M, Yamashita H, Ueta Y: Adrenomedullin regulates blood-brain barrier functions in vitro. *Neuroreport* 2001, 12:4139–4142
 41. Srinivasan B, Kolli AR, Esch MB, Abaci HE, Shuler ML, Hickman JJ: TEER measurement techniques for in vitro barrier model systems. *J Lab Autom* 2015, 20:107–126
 42. Joussen AM, Poulaki V, Le ML, Koizumi K, Esser C, Janicki H, Schraermeyer U, Kociok N, Fauser S, Kirchhof B, Kern TS, Adamis AP: A central role for inflammation in the pathogenesis of diabetic retinopathy. *FASEB J* 2004, 18:1450–1452
 43. Hayden MS, Ghosh S: Signaling to NF-kappaB. *Genes Dev* 2004, 18: 2195–2224
 44. New WHO statistics highlight increases in blood pressure and diabetes, other noncommunicable risk factors. *Cent Eur J Public Health* 2012, 20:134, 149
 45. Kirsch S, Iroku-Malize T: Eye conditions in older adults: diabetic retinopathy. *FP Essent* 2016, 445:29–37
 46. Antonetti DA, Klein R, Gardner TW: Diabetic retinopathy. *N Engl J Med* 2012, 366:1227–1239
 47. Davidson JA, Ciulla TA, McGill JB, Kles KA, Anderson PW: How the diabetic eye loses vision. *Endocrine* 2007, 32:107–116
 48. Bhagat N, Grigorian RA, Tutela A, Zarbin MA: Diabetic macular edema: pathogenesis and treatment. *Surv Ophthalmol* 2009, 54:1–32
 49. Curtis TM, Gardiner TA, Stitt AW: Microvascular lesions of diabetic retinopathy: clues towards understanding pathogenesis? *Eye (Lond)* 2009, 23:1496–1508
 50. Scholl S, Augustin A, Loewenstein A, Rizzo S, Kupperman B: General pathophysiology of macular edema. *Eur J Ophthalmol* 2011, 21 Suppl 6:S10–S19
 51. Tolentino MJ, Miller JW, Gragoudas ES, Jakobiec FA, Flynn E, Chatzistefanou K, Ferrara N, Adamis AP: Intravitreal injections of vascular endothelial growth factor produce retinal ischemia and microangiopathy in an adult primate. *Ophthalmology* 1996, 103: 1820–1828
 52. Abid MR, Schoots IG, Spokes KC, Wu SQ, Mawhinney C, Aird WC: Vascular endothelial growth factor-mediated induction of manganese superoxide dismutase occurs through redox-dependent regulation of forkhead and IkappaB/NF-kappaB. *J Biol Chem* 2004, 279: 44030–44038
 53. Arnott C, Punna-Moorthy G, Tan J, Sadeghipour S, Bursill C, Patel S: The vascular endothelial growth factor inhibitors Ranibizumab and Aflibercept markedly increase expression of atherosclerosis-associated inflammatory mediators on vascular endothelial cells. *PLoS One* 2016, 11:e0150688
 54. Pearson T, Shultz LD, Lief J, Burzenski L, Gott B, Chase T, Foreman O, Rossini AA, Bottino R, Trucco M, Greiner DL: A new immunodeficient hyperglycaemic mouse model based on the Ins2A-kita mutation for analyses of human islet and beta stem and progenitor cell function. *Diabetologia* 2008, 51:1449–1456
 55. Kim W, Moon SO, Lee S, Sung MJ, Kim SH, Park SK: Adrenomedullin reduces VEGF-induced endothelial adhesion molecules and adhesiveness through a phosphatidylinositol 3'-kinase pathway. *Arterioscler Thromb Vasc Biol* 2003, 23:1377–1383
 56. Hombrebueno JR, Chen M, Penalva RG, Xu H: Loss of synaptic connectivity, particularly in second order neurons is a key feature of diabetic retinal neuropathy in the Ins2Akita mouse. *PLoS One* 2014, 9:e97970
 57. Shen WY, Lai CM, Graham CE, Binz N, Lai YK, Eade J, Guidolin D, Ribatti D, Dunlop SA, Rakoczy PE: Long-term global retinal microvascular changes in a transgenic vascular endothelial growth factor mouse model. *Diabetologia* 2006, 49:1690–1701
 58. Ali Rahman IS, Li CR, Lai CM, Rakoczy EP: In vivo monitoring of VEGF-induced retinal damage in the Kimba mouse model of retinal neovascularization. *Curr Eye Res* 2011, 36:654–662
 59. Ishida S, Usui T, Yamashiro K, Kaji Y, Ahmed E, Carrasquillo KG, Amano S, Hida T, Oguchi Y, Adamis AP: VEGF164 is

- proinflammatory in the diabetic retina. *Invest Ophthalmol Vis Sci* 2003, 44:2155–2162
60. Hombrebueno JR, Luo C, Guo L, Chen M, Xu H: Intravitreal injection of normal saline induces retinal degeneration in the C57BL/6J mouse. *Transl Vis Sci Technol* 2014, 3:3
 61. Minamino N, Kikumoto K, Isumi Y: Regulation of adrenomedullin expression and release. *Microsc Res Tech* 2002, 57:28–39
 62. Zhou M, Simms HH, Wang P: Adrenomedullin and adrenomedullin binding protein-1 attenuate vascular endothelial cell apoptosis in sepsis. *Ann Surg* 2004, 240:321–330
 63. Kato H, Shichiri M, Marumo F, Hirata Y: Adrenomedullin as an autocrine/paracrine apoptosis survival factor for rat endothelial cells. *Endocrinology* 1997, 138:2615–2620
 64. Fernandez-Sauze S, Delfino C, Mabrouk K, Dussert C, Chinot O, Martin PM, Grisoli F, Ouafik L, Boudouresque F: Effects of adrenomedullin on endothelial cells in the multistep process of angiogenesis: involvement of CRLR/RAMP2 and CRLR/RAMP3 receptors. *Int J Cancer* 2004, 108:797–804
 65. Nagaya N, Mori H, Murakami S, Kangawa K, Kitamura S: Adrenomedullin: angiogenesis and gene therapy. *Am J Physiol Regul Integr Comp Physiol* 2005, 288:R1432–R1437
 66. Oehler MK, Hague S, Rees MC, Bicknell R: Adrenomedullin promotes formation of xenografted endometrial tumors by stimulation of autocrine growth and angiogenesis. *Oncogene* 2002, 21:2815–2821
 67. Ichikawa-Shindo Y, Sakurai T, Kamiyoshi A, Kawate H, Iinuma N, Yoshizawa T, Koyama T, Fukuchi J, Iimuro S, Moriyama N, Kawakami H, Murata T, Kangawa K, Nagai R, Shindo T: The GPCR modulator protein RAMP2 is essential for angiogenesis and vascular integrity. *J Clin Invest* 2008, 118:29–39
 68. Zhang W, Liu H, Al-Shabrawey M, Caldwell RW, Caldwell RB: Inflammation and diabetic retinal microvascular complications. *J Cardiovasc Dis Res* 2011, 2:96–103