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

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-κB 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.

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 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.), SENSIN 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.

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moderate, and severe stages) and proliferative DR (PDR; advanced stage) with neovascularization, preretinal or vitreous hemorrhage, and fibrovascular proliferation. 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. 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, exudative age-related macular degeneration, retinal vein occlusion, and retinopathy of prematurity. 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. Therefore, identification of other therapeutic targets involved in controlling vascular integrity would be desirable.

Originally isolated from human pheochromocytoma, adrenomedullin (ADM) is a vasodilating polypeptide whose expression is up-regulated under ischemic conditions. We previously showed that ADM knockout (Adm−/−) mice die in utero because of vascular structural abnormalities, 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. Moreover, ADM is now known to be widely distributed in various 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.

Increased plasma ADM levels are observed in diabetes, 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. Likewise, plasma ADM levels are elevated in patients with type 2 diabetes and DR. Vitreous ADM levels are also elevated in patients with DR, and plasma or vitreous ADM levels are reportedly related to the severity and stability of DR. These clinical data strongly implicate ADM in the pathogenesis of DR, although the specific function of ADM in DR remains to be clarified.

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-Ins2Akita/J (Akita) mouse, which is a genetic model of type 1 diabetes, or the trVEGF029 (Kimba) mouse, which is a homozygous transgenic mouse line exhibiting photoreceptor (rhodopsin)-specific overexpression of human VEGF-165 (hVEGF165). More recently, Akimba mice (Ins2Akita VEGF+/−) were generated from Kimba (VEGF+/−) and Akita mice. 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 Ins2Akita mice on a C57BL/6J background (C57BL/6-Ins2Akita/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 (Ins2Akita 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 Ins2Akita allele are established as suitable models of diabetic complications. After genotyping, blood glucose levels were measured to ensure diabetic status. Genotyping for the Ins2Akita allele was performed using PCR in a Veriti 96-Well Thermal Cycler (Thermo Fisher Scientific, Waltham, MA) with primers 5′-TGGTCCCACATATGCACATG-3′ (forward) and 5′-TGCTGATGCCCTGGCCTGCT-3′ (reverse). We previously showed that ADM knockout (Adm−/−) mice die in utero because of vascular structural abnormalities, 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. Moreover, ADM is now known to be widely distributed in various 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.

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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 a primers 5′-TGGTCCCACATATGCACATG-3′ (forward) and 5′-TGCTGATGCCCTGGCCTGCT-3′ (reverse), and with a restriction enzyme digestion protocol described by The Jackson Laboratory (Bar Harbor, ME) in protocol Ins2Akita.

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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 previously with some modifications. We used an endoscope coupled to a 5-cm-long otoscope with a 3-mm outer diameter (1218AA; Karl Storz, Tuttingen,
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 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 μL/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 isoelectin 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 ×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. 5 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 μL/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).19 Twenty microliters of sample (5 μL of aqueous humor + 15 μL of dH2O) 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.
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Table 1  Primers Used for Real-Time PCR

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>h VEGF-165 forward</td>
<td>5'-CTACCTCCACCAGGCAAGG-3'</td>
</tr>
<tr>
<td>h VEGF-165 reverse</td>
<td>5'-CCGCAGCTCTGCAGATAG-3'</td>
</tr>
<tr>
<td>m VEGF-A forward</td>
<td>5'-GTCCATGCTTGCAGGAGGT-3'</td>
</tr>
<tr>
<td>m VEGF-A reverse</td>
<td>5'-GCTCAATTGGCAGTCCACTG-3'</td>
</tr>
<tr>
<td>m MCP-1 forward</td>
<td>5'-CACCGGCTCTGCTGAG-3'</td>
</tr>
<tr>
<td>m MCP-1 reverse</td>
<td>5'-TGAATTGGATGGTCTTGGTCC-3'</td>
</tr>
<tr>
<td>m IL-1β forward</td>
<td>5'-AGGCACTGCTGAGCCGAGT-3'</td>
</tr>
<tr>
<td>m IL-1β reverse</td>
<td>5'-CAGCCCGTAGTGCTGCAAG-3'</td>
</tr>
<tr>
<td>m p22 phox forward</td>
<td>5'-GCCCATTGAGGTGGAGAGCTTTC-3'</td>
</tr>
<tr>
<td>m p22 phox reverse</td>
<td>5'-CTACAGGCTCCGAATGAAACAC-3'</td>
</tr>
<tr>
<td>m p47 phox forward</td>
<td>5'-ATCCTATCTGGAGCCCTTGA-3'</td>
</tr>
<tr>
<td>m p47 phox reverse</td>
<td>5'-TCCATTGAGGTGGAGAGCTTTC-3'</td>
</tr>
<tr>
<td>m p67 phox forward</td>
<td>5'-AGATAGCAAATCGGCTGACG-3'</td>
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<tr>
<td>m p67 phox reverse</td>
<td>5'-CAGCCCGTAGTGCTGCAAG-3'</td>
</tr>
<tr>
<td>m eNOS forward</td>
<td>5'-AGGCACTGCTGAGCCGAGT-3'</td>
</tr>
<tr>
<td>m eNOS reverse</td>
<td>5'-TTCTCCAGGGTCCTACCAGGC-3'</td>
</tr>
</tbody>
</table>

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-μg 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 StepOnePlus Real-Time PCR System (Thermo Fisher Scientific) with SYBR green (Toyobo, Osaka, Japan) or TaqMan probe (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, Germany), 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.

Western Blot Analysis

Retinal tissue was lyzed in an ice-cold RIPA Lysis Buffer System (Santa Cruz Biotechnology, Santa Cruz, CA) supplemented with Proteinase Inhibitor cocktail (Roche Applied Science, Mannheim, Germany). Samples of the resultant lysate were subjected to SDS-PAGE, and the resolved proteins were transferred to nitrocellulose membranes (GE Healthcare). The detection of NF-κB and the secondary antibodies were used in 1:4000 dilution. Western blot analysis used 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; Direct Co., Cupertino, CA). The delivery rate was 26 μg/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 μL) 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 μL of PBS. After the procedure, the mice were administered oxiflaxacin 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 using RT² Profiler Array Data Analysis Software version 3.5 (Qiagen).
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 \(1.0 \times 10^5\) 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 \(\mu\)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 \(\mu\)m polyethylene terephthalate (Merck Millipore) was used. TR-iBRB cells were plated at a density of \(1.0 \times 10^5\) 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 4°C for 60 minutes. After washing the cells three times for 3 minutes each with PBS, they were incubated with anti–NF-κB 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.

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**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-κB p65 and IκBα Detection

Whole-cell extracts were prepared from $1 \times 10^3$ 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-κB p65 and IκBα 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-κB p65 and phospho-IκBα 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 ± 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). 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 fluorescent 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 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 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.
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
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). 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 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 ± 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; MCP, monocyte chemoattractant protein; PBS, phosphate-buffered saline; TNF, tumor necrosis factor; 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 overexpression 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. 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 coordinate 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 VEGF-Induced Retinopathy**

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.
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 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 ± SEM. n = 7 in each group (C), n = 5 in each group (F). *P < 0.05 (t-test). W, week.
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 lower chamber of the cell monolayer. The permeability index was expressed in terms of 10^(-6) cm/s.

**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 ± SEM. *P < 0.05, **P < 0.001 (t-test).

**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^{-7} to 10^{-8} 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 ± SEM. *P < 0.05 in each group (A and B); n = 3 in each group (C). *P < 0.05 phosphate-buffered saline (PBS) versus VEGF (one-way analysis of variance) (B). Scale bars = 40 μm.
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 (Figure 8B). In the 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.

ADM Suppresses VEGF-Induced Inflammation

Stimulation of TR-iBRB cells with VEGF led to upregulated 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, 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...
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, and DR is a leading cause of blindness, estimated to affect between 28% and 40% of diabetic patients older than 40 years. Microvascular sequelae are present in 25% of diabetic patients and are the primary mechanism responsible for DR. 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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 IκB/NF-κB,\textsuperscript{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.\textsuperscript{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-κB signaling.\textsuperscript{54} ADM also reportedly inhibits VEGF-induced expression of inflammatory molecules through the phosphatidylinositol 3'-kinase–Akt pathway.\textsuperscript{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.\textsuperscript{56} By contrast, Akiba mice showed advanced PDR, including diffuse vascular leakage, edema, proliferative changes, and retinal detachment.\textsuperscript{53} Kimba mice reportedly exhibit pathologic changes to the retinal vasculature at 3 to 4 weeks of age, and stable retinopathy persists for 3 months.\textsuperscript{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,\textsuperscript{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,\textsuperscript{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.\textsuperscript{20–22} Moreover, vitreous ADM levels are also elevated in DR patients,\textsuperscript{23–27} and vitreous ADM is higher in DR patients with DME.\textsuperscript{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.\textsuperscript{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,\textsuperscript{51} 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.\textsuperscript{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\textsuperscript{−/−}) die \textit{in utero} because of vascular structural abnormalities,\textsuperscript{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\textsuperscript{−/−} mice exhibited a
lethal phenotype nearly identical to that of Adm−/− mice. 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. DME is caused by vascular leakage resulting from compromise of the blood-retinal barrier. As with other retinal diseases, DME is multifactorial, although inflammation likely plays a substantial role. DME can be thought of as a state of chronic low-grade inflammation that accompanies vascular leakage. 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.

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