Pigment epithelium-derived factor (PEDF) inhibits advanced glycation end product-induced retinal vascular hyperpermeability by blocking reactive oxygen species-mediated vascular endothelial growth factor (VEGF) expression

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Running Title: PEDF and diabetic retinopathy

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Pigment epithelium-derived factor (PEDF) is the most potent inhibitor of angiogenesis, suggesting that loss of PEDF contributes to proliferative diabetic retinopathy. However, the role of PEDF against retinal vascular hyperpermeability, the
characteristic feature of early diabetic retinopathy, remains to be elucidated. Since advanced glycation end products (AGEs) elicit vasopermeability response, we investigated here whether and how PEDF could inhibit the AGE-signaling to vascular hyperpermeability. Intravenous administration of AGEs to normal rats not only increased retinal vascular permeability by stimulating vascular endothelial growth factor (VEGF) expression, but also decreased retinal PEDF levels. Simultaneous treatments with PEDF inhibited the AGE-elicited VEGF-mediated permeability by down-regulating mRNA levels of p22phox and gp91phox, membrane components of NADPH oxidase and subsequently decreasing retinal levels of an oxidative stress marker, 8-hydroxydeoxyguanosine. In vitro, PEDF also inhibited the AGE-induced vascular hyperpermeability evaluated by transendothelial electrical resistance by suppressing VEGF expression. Furthermore, PEDF decreased reactive oxygen species (ROS) generation in AGE-exposed microvascular endothelial cells (ECs) by suppressing NADPH oxidase activity via down-regulation of mRNA levels of p22phox and gp91phox. This led to blockade of the AGE-elicited Ras activation and NF-κB-dependent VEGF gene induction in ECs. These results indicate that the central mechanism for PEDF inhibition of the AGE signaling to vascular permeability is by suppression of NADPH oxidase-mediated ROS generation and subsequent VEGF expression. Substitution of PEDF may offer a promising strategy for halting the development of diabetic retinopathy.
Diabetic retinopathy is one of the miserable microvascular complications in diabetes and is a leading cause of acquired blindness among the people of occupational age (1). Chronic hyperglycemia is a major initiator of diabetic retinopathy. Two recent large prospective clinical studies have shown that intensive blood glucose control reduces microvascular complications among patients with diabetes (2,3). However, strict control of hyperglycemia is often difficult to maintain and may increase the risk of severe hypoglycemia in diabetic patients. Therefore, to develop novel therapeutic strategies that specifically target diabetic retinopathy is desired for patients with diabetes.

Various hyperglycemia-induced metabolic and hemodynamic derangements have been reported to contribute to the characteristic histopathological changes observed in diabetic retinopathy (4). Among them, advanced glycation end products (AGEs), the senescent macroprotein derivatives, whose formation and accumulation occur at an accelerated rate in diabetes (5-7), have been strongly implicated in the pathogenesis of diabetic vascular complications (8-15). Indeed, we, along with others, have previously shown that AGEs could elicit a brisk angiogenic response, at least in part, by inducing autocrine production of vascular endothelial growth factor (VEGF), which is an important mediator in the development and progression of diabetic retinopathy (16-18). AGEs exert pleiotropic actions on cells by inducing the generation of intracellular reactive oxygen species (ROS) (19). ROS in turn activate the Ras proto-oncogene and its downstream effectors that are important for both proliferative and differentiative responses.

Pigment epithelium-derived factor (PEDF) is a glycoprotein that belongs to the superfamily of serine protease inhibitors (20). It was first purified from the conditioned media of human retinal pigment epithelial cells as a factor with potent neuronal differentiating activity (20). Recently, PEDF has been shown to be a highly effective inhibitor of angiogenesis in cell culture and animal models. PEDF inhibits the growth and migration of cultured endothelial cells (ECs), and it potently suppresses ischemia-induced retinal neovascularization (21,22). PEDF levels in aqueous humor or vitreous are decreased in diabetic patients, especially with proliferative retinopathy (23-25). These observations suggest that the loss of PEDF activity in the eye may contribute to the pathogenesis of proliferative
diabetic retinopathy. However, the protective role of PEDF against retinal vascular hyperpermeability, the characteristic feature of early diabetic retinopathy, remains to be elucidated. In this study, we have investigated whether PEDF could inhibit the AGE-induced retinal vascular hyperpermeability and the mechanism by which it might achieve this beneficial effect.

MATERIALS AND METHODS

Materials
Diphenylene iodonium (DPI), lucigenin and NADPH were purchased from Sigma (St. Louis, MO). Polyclonal antibodies (Abs) against rat VEGF were purchased from R&D systems (Genzyme-Teche, Minneapolis, MN). Protease inhibitor cocktails were from Nakalai Tesque (Kyoto, Japan).

Purification of PEDF Proteins
PEDF proteins were prepared and purified as described previously (12). SDS-PAGE analysis of purified PEDF proteins revealed a single band with a molecular weight of about 50 kDa, which showed positive reactivity with monoclonal Ab against human PEDF (Transgenic, Kumamoto, Japan).

Preparations of AGES
AGE-bovine serum albumin (BSA) was prepared as described previously (12). Briefly, BSA (50 mg/ml) was incubated under sterile conditions with 0.1 M D-glyceraldehyde in 0.2 M NaPO₄ buffer (pH 7.4) for 7 days. Then unincorporated sugars were removed by dialysis against phosphate-buffered saline. Control non-glycated BSA was incubated in the same conditions except for the absence of reducing sugars. Preparations were tested for endotoxin using Endospecy ES-20S system (Seikagaku Co., Tokyo, Japan); no endotoxin was detectable.

Preparations of AGE-Rich Serum Fractions from Diabetic Patients on Hemodialysis
Serum AGE fractions were obtained from normal volunteers (Normal) and diabetic patients with hemodialysis (DM-AGEs) as described previously (26). Briefly, 10 ml of serum from each of 4 normal individuals and from 11 type 2 diabetic patients with end-stage renal disease on hemodialysis were concentrated by lyophilization and dissolved in 2 ml of distilled water. These solutions were applied to a Sephacryl S-200 column (1.5 x 110 cm), which was equilibrated with PBS (pH 7.4) and eluted with the same buffer (fraction size: 1.5 ml, flow rate: 10 ml/h) in a cold room. Each fraction was monitored for absorbance at 280 nm and the AGE concentration of each fraction was measured by a competitive ELISA as described below. Preparations were passed through Zeta-Pore filter to remove endotoxin. No endotoxin was detectable. AGE-rich serum fractions obtained from diabetic patients on hemodialysis and normal volunteers contained 176.1 and 28.5 μg/ml AGES, respectively. Clinical characteristics of 11 type 2 diabetic patients were shown in Table 1.

Enzyme-Linked Immunosorbent Assay (ELISA) for AGES
Measurement of AGES was performed with a competitive ELISA as described previously (27). Briefly, 96 well microtiter plates were coated with 0.1 μg/ml AGE-BSA. Then, test samples (50 μl) were added to each well as a competitor for 50 μl of polyclonal Abs directed against AGE-BSA (1:1000), followed by incubation for 2 h at room temperature with gentle shaking on a horizontal rotary shaker. After incubating each well with alkaline phosphatase-conjugated anti-rabbit IgG, p-nitrophenyl phosphate was added as a colorimetric substrate. Then the plate was read at 405 nm using a microplate reader.

In Vitro-Prepared AGE Treatments of Normal Rats
Nine wk-old normoglycemic Sprague-Dawley (SD) rats were injected intravenously with 1 mg AGE-BSA or non-glycated BSA in the presence or absence of 10 μg PEDF proteins or 10 μg Abs against rat VEGF every day for up to 10 days. The rats were sacrificed 1-2 h after injection on the final day. This AGE administration increases serum AGE levels by about 2-fold, compared with non-glycated BSA injection (33.4±1.6 vs 18.7±0.5 μg/ml). We have recently found that serum level of AGES in diabetic rats (Goto-Kakizaki rats at 14 weeks old) was 32.8±7.1 μg/ml. Therefore, the serum AGE concentrations...
obtained by the AGE injection were comparable to those of diabetic rats. All animal procedures were conducted according to the guidelines provided by the Kurume University Institutional Animal Care and Use Committee under an approved protocol.

**Leakage of FITC-Conjugated Dextran from Retinal Vasculature**

Leakage of FITC-conjugated dextran from retinal vasculature was determined by the method of Stitt et al (18). Briefly, rats were deeply anesthetized, and then FITC-conjugated dextran (40 kDa, Sigma) was injected into the inferior vena cava. After the tracer was allowed to circulate, the eyes were enucleated and immediately fixed in 4 % paraformaldehyde (Sigma). The retinas were imaged by a laser-scanning confocal microscope.

**Quantification of Blood Retinal Barrier (BRB) Breakdown**

BRB breakdown quantification was determined by the method of Adamis et al (28). Briefly, after deep anesthesia, the rats received intravenous injection with FITC-conjugated dextran (4.4 kDa, Sigma). After 10-15 minutes, a blood sample was collected and then each rat was perfused with PBS. After perfusion, the retinas were carefully removed, weighted, and homogenized to extract the FITC-conjugated dextran. BRB breakdown was calculated using the following equation:

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\text{Retinal FITC-dextran (mg)/retinal weight (g) = } \frac{\text{Plasma FITC-dextran concentration (mg/mL) x circulation time (hour)}}{\text{Retinal FITC-dextran (mg)/retinal weight (g)}}
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**Effects of AGE-Rich Serum Fractions on Retinal Vascular Permeability**

Nine wk-old SD rats were injected intravenously with AGE-rich serum fractions derived from normal volunteers (Normal) or diabetic patients with hemodialysis (DM-AGEs) in the presence or absence of 10 µg PEDF proteins or 77 µg polyclonal anti-human PEDF Abs. After every day-injection for 5 days, the rats were sacrificed and retinal permeability and BRB breakdown were analyzed.

**Cells**

Human adult skin microvascular ECs were cultured in endothelial basal medium supplemented with 5% fetal bovine serum, 0.4% bovine brain extracts, 10 ng/ml human epidermal growth factor and 1 µg/ml hydrocortisone according to the supplier’s instructions (Clonetics Corp., San Diego, CA). AGE treatment was carried out in a medium lacking epidermal growth factor and hydrocortisone. Cells at 3-5 passages were used for the experiments.

**Quantitative Real-Time Reverse Transcription–Polymerase Chain Reactions (RT-PCR)**

Poly(A) RNAs were isolated from ECs or enucleated eyes as described previously (16). Quantitative real-time RT-PCR was performed using Assay-on-Demand and TaqMan 5 fluorogenic nuclease chemistry (Applied Biosystems, Foster city, CA) according to the manufacturer’s recommendation. IDs of primers for human p22phox, human gp91phox, human VEGF, rat p22phox, rat gp91phox, and rat VEGF gene were Hs00164370_m1, Hs00166163_m1, Hs00173626_m1, Rn00577357_m1, Rn00576710_m1, and Rn00582935_m1, respectively.

**Immunohistochemistry**

Rat eyes were removed and fixed for 1 day in 4 % paraformaldehyde. Then the eyes were embedded in paraffin wax for sectioning. Five µm paraffin sections were incubated with polyclonal Abs raised against human PEDF (4 µg/ml; Santa Cruz Biotechnology, Santa Cruz, CA) or monoclonal Abs raised against 8-hydroxydeoxyguanosine (8-OHdG) (10 µg/ml; Japan Institute for the Control of Aging, Shizuoka, Japan). After exposure to peroxidase-labelled secondary anti-rabbit Abs, the sections were counterstained with methyl green for visualizing PEDF or 8-OHdG immunoreactivity. The immunoreactivity was measured with microcomputer-assisted NIH Image.

**Measurement of Transendothelial Electrical Resistance (TER)**

Effects of PEDF on barrier function of ECs were assessed by measurement of TER using electric cell substrate impedance sensing (Applied Biophysics) according to the method of Becker et al (29). Briefly, the cells were seeded onto gold microelectrodes (Applied Biophysics) and grown to confluence for 2 days. Cells were washed three times with serum-free endothelial basal medium and then were connected to the impedance sensing system to measure the TER baseline. The applied alternating current (1 µA) was clamped so that impedance (resistance) was directly related to changes in voltage, which was measured with a...
locked-in amplifier. Data from the electrical resistance experiments (ohms) were obtained over the experimental time course at 5-min intervals. Resistance values for each microelectrode were normalized as the ratio of measured resistance to baseline resistance and plotted as a function of time.

**NADPH Oxidase Assay**
ECs were treated with 100 μg/ml of AGE-BSA or non-glycated BSA in the presence or absence of 10 nM PEDF for 24 h, and then the cells were suspended in homogenization buffer (20 mM Hepes, pH 7.0, 100 mM KCl, and 1 mM EDTA containing protease inhibitor cocktails). NADPH oxidase activity of the cell homogenate was measured by luminescence assay in 50 mM phosphate buffer, pH 7.0, containing 1 mM EGTA, 150 mM sucrose, 5 μM lucigenin as the electron acceptor, and 100 μM NADPH as a substrate according to the methods of Griendling et al (30).

**Preparations of Abs Directed against In Vitro-Prepared AGE-BSA**
Polyclonal Abs directed against in vitro-modified AGE-BSA were prepared as described previously (27). We have previously shown that the Abs did not cross-react with several structurally identified AGE-modified BSA including pyrraline-BSA, pentosidine-BSA, argpyrimidine-BSA, 3-deoxyglucosone imidazolone-BSA, carboxymethyllysine-BSA, carboxyethyllysine-BSA, glyoxal-lysine dimmer, or methyglyoxal-lysine dimmer (13,27).

**Preparations of Antiserum Directed against Receptor for AGEs (RAGE)**
Antiserum directed against human RAGE for neutralizing assays, which recognizes the amino acid residues 167 to 180 of human RAGE protein, was prepared as described previously (31).

**Intracellular ROS Generation**
ECs were treated with various concentrations of AGE-BSA or non-glycated BSA in the presence or absence of 10 nM PEDF, 50 nM DPI, 10 μg/ml Abs directed against AGEs, or 0.1 % anti-RAGE serum for 24 h. The intracellular formation of ROS was detected by using the fluorescent probe CM-H_2DCFDA (Molecular Probes Inc., Eugene, OR) as described previously (15).

**Superoxide Generation**
ECs were treated with serum AGE fractions in the presence or absence of 10 nM PEDF for 24 h, and then the cells were incubated with phenol red free DMEM containing 3 μmol/l dihydroethidium (DHE) (Molecular Probes Inc., Eugene, OR). After 30 minutes, fluorescence intensity was measured, and the cells were imaged by a laser-scanning confocal microscope.

**Assay for Ras Activation**
ECs were treated with 100 μg/ml of AGE-BSA or non-glycated BSA in the presence or absence of 10 nM PEDF or 1 mM NAC for 24 h. Ras activity then was measured using a Ras Activation Assay Kit (Upstate Biotechnology Inc., Lake Placid, NY) following the manufacturer's instructions.

**Transfection of Dominant-Negative Mutant Vector**
ECs were transiently transfected with a dominant-negative human Ras mutant (DN-Ras) vector or an empty vector (Mock) as described previously (17).

**Measurement of NF-κB Activity**
ECs were transiently transfected with plasmids containing the NF-κB promoter attached upstream to the luciferase reporter gene. Luciferase activity was measured as described previously (17).

**Statistical Analysis**
All values were presented as means ± SE. One-way ANOVA followed by the Scheffe F test, was performed for statistical comparisons. P<0.05 was considered significant.

**RESULTS**

**Anti-Vasopermeability Effects of PEDF In Vivo**
We used glyceraldehyde-modified AGE-BSA for the present experiments because we have previously shown that this type of AGEs could elicit a brisk angiogenic response by inducing autocrine production of VEGF, also known as a vascular permeability factor (17,32,33).
We first examined whether intravenous administration of \textit{in vitro}-prepared AGE-BSA to normal rats increased retinal vascular leakage \textit{in vivo}. As shown in Figure 1A, AGE-BSA increased retinal vascular permeability compared to non-glycated BSA. BRB function was also disturbed by the treatment with AGES. The quantitative pooled data of BRB breakdown are shown in Figure 1B. Moreover, in order to evaluate the pathophysiological relevance of the experiments using \textit{in vitro}-prepared AGES, we studied whether AGE-rich serum fractions derived from diabetic patients on hemodialysis (DM-AGEs) could elicit the same biological response. As shown in Figure 1C and 1D, DM-AGEs also increased retinal vascular permeability and induced BRB breakdown.

Next, we examined the role of VEGF for the AGE-induced vascular hyperpermeability in two different ways; VEGF gene expression and effects of anti-VEGF Abs. VEGF mRNA levels were increased in the eye of AGE-treated rats (Figure 1E), while anti-VEGF Abs completely suppressed the AGE-induced retinal vascular hyperpermeability (Figure 1A). These observations indicate the central role of VEGF in the AGE-induced retinal vascular leakage.

PEDF is a potent anti-angiogenic factor; it inhibits the VEGF-induced proliferation and migration of ECs (21,22). Since VEGF is also known as a vascular permeability factor (32,33), we examined the involvement of PEDF and its therapeutic efficacy in the AGE-induced vascular hyperpermeability. Immunohistochemical analysis revealed that administration of AGE-BSA to normal rats decreased expression levels of PEDF in the retina, compared with that of non-glycated BSA; PEDF immunoreactivity in the ganglion cell layer and in the inner plexiform layer of AGE-injected rats was decreased to about 70% of that of non-glycated BSA-treated rats (Figure 1F). Furthermore, simultaneous treatments with PEDF inhibited the up-regulation of VEGF mRNA levels in the eye of AGE-injected rats (Figure 1E). In addition, PEDF was found to block both the \textit{in vitro}-prepared AGE-BSA- and DM-AGE-induced retinal vascular hyperpermeability and BRB breakdown (Figure 1A-1D) and the effects of PEDF were reversed by the treatments with PEDF Abs (Figure 1C). Taken together, our data indicate that PEDF could block the AGE-induced retinal hyperpermeability and BRB breakdown by suppressing VEGF expression.
Because it has been demonstrated that NADPH oxidase plays an important role in the AGE-elicited ROS generation and subsequent gene expression in cultured ECs (34), we further examined whether intravenous administration of PEDF suppressed the up-regulation of mRNA levels for p22phox and gp91phox, key components of NADPH oxidase with respect to its enzymatic activity (35), in the eye of AGE-treated rats. AGEs up-regulated mRNA levels of these membrane components of NADPH oxidase in the eye, which was suppressed by PEDF treatments (Figure 1G). Moreover, immunohistochemistry of 8-OHdG, a sensitive indicator of oxidative damage to DNA, showed intense staining in the nuclei of cells in the inner and outer plexiform layers of AGE-treated retina, which was also blocked by simultaneous PEDF treatments (Figure 1H). These observations suggest that PEDF could inhibit the AGE-induced retinal vascular hyperpermeability by suppressing VEGF induction via inhibition of NADPH oxidase expression and ROS generation.

In addition, the present findings suggest that AGE infusion could down-regulate retinal PEDF levels, at least in part, via oxidative stress generation because we have recently found that AGE-BSA or H$_2$O$_2$ suppresses PEDF gene expression in microvascular ECs and that antioxidant N-acetylcysteine restores high glucose-induced decrease in PEDF gene expression in cultured retinal pericytes (36,37).

**Anti-Vasopermeability Effects of PEDF In Vitro**

We next investigated whether PEDF inhibited the AGE-induced barrier dysfunction of microvascular ECs *in vitro*. In this experiment, we evaluated TER as an indicator of EC barrier function (28). Reports vary on serum AGE levels in diabetic patients (38-41). The difference of AGE epitopes to target, chemical nature of AGEs used as a standard, or methods for the measurement of AGEs may account for the discrepancies of the results. In our ELISA system, serum concentration of AGEs in diabetic patients was about 30-50 µg/ml, and their levels were increased two- to three-fold in diabetic patients with uremia (41,42). So, we chose the concentration of *in vitro*-prepared AGEs at 100 µg/ml for the following experiments. As shown in Figure 2A, TER in AGEs-exposed ECs was significantly decreased in a time-dependent manner, reaching a nadir of 60% of non-glycated BSA-treated cells 200 minutes after the treatments. PEDF significantly inhibited the decrease of TER in AGE-treated ECs. Thus, *in...
we were able to reproduce the inhibitory effects of PEDF on the AGE-induced barrier dysfunction shown in vivo. AGE treatments did not induce apoptotic cell death of ECs during the experiment periods (data not shown). Therefore, it is unlikely that increased apoptotic death of ECs could be involved in monolayer permeability responses elicited by AGEs.

PEDF also inhibited the AGE-induced up-regulation of VEGF mRNA levels in cultured ECs (Figure 2B). These observations suggest that, as the case in vivo, PEDF could exert anti-vasopermeability effects on AGE-exposed ECs by suppressing VEGF expression. In order to further investigate the molecular mechanism by which PEDF inhibited the AGE-induced vascular permeability, we conducted the following experiments using microvascular ECs.

**Molecular Mechanism for the Suppression by PEDF of the Vasopermeability Effects of AGES**

In vivo we showed that PEDF inhibited up-regulation of mRNA levels for p22phox and gp91phox. Therefore, we first addressed this issue in vitro. As shown in Figure 3A, AGES up-regulated mRNA levels of p22phox and gp91phox, which was significantly blocked by PEDF. Moreover, PEDF inhibited the increase of NADPH activity induced by AGES (Figure 3B). Ten nM PEDF alone did not affect the NADPH oxidase activity in ECs (data not shown).

AGEs exert a pleiotropic action on multiple cell functions through the generation of ROS (13-15). Because ROS generation is the downstream of NADPH oxidase, it is plausible that PEDF could inhibit the AGE signaling to permeability by blocking ROS generation. To address this issue, we next investigated whether PEDF blocked the AGE-induced ROS generation in ECs. As shown in Figure 3C, AGES increased intracellular ROS generation in a dose-dependent manner. PEDF or DPI, an inhibitor of NADPH oxidase, inhibited the generation of ROS in ECs exposed to AGES; 10 nM PEDF completely inhibited the AGE-induced increase in ROS generation in ECs. In addition, Abs directed against AGES or anti-RAGE serum completely blocked the AGE-induced ROS generation in ECs, thus suggesting the involvement of the AGE-RAGE interaction in ROS generation. We also confirmed here that PEDF exerted antioxidative effects on DM-AGE-exposed ECs. DM-AGEs elicited superoxide generation in ECs as did in vitro-prepared AGES, which was also blocked by 10 nM PEDF (Figure 3D). Taken together, these results suggest that down-
regulation of p22phox and gp91phox mRNAs might be a central mechanism for the antioxidative effects of PEDF on AGE-exposed ECs.

Ras has been proposed as a key regulator of the signaling cascade triggered by oxidative stress (43,44), and is also required for VEGF expression in various cell types (45,46). Accordingly, we next examined whether Ras could be a downstream target for PEDF. A Ras-GTP pulldown assay revealed that AGEs significantly activated Ras in ECs, which was inhibited by PEDF (Figure 3E).

Since we have previously shown that AGEs stimulated VEGF gene induction in ECs through transcriptional activation of NF-κB (17,47), we next investigated the possibility whether Ras could mediate the NF-κB-dependent VEGF gene induction elicited by AGEs and whether it was the pathway that was shut down by PEDF. PEDF as well as dominant-negative human Ras mutant (DN-RasS17N) overexpression was found to inhibit the AGE-induced increase in NF-κB promoter activity in AGE-exposed ECs (Figures 3F and 3G, respectively). These observations suggest that the NADPH oxidase-mediated ROS-induced Ras activation is crucial for the AGE signaling to VEGF induction in ECs, which is also blocked by PEDF.

DISCUSSION

In the present study, we demonstrated for the first time that PEDF could inhibit the AGE-elicited retinal vascular hyperpermeability by suppressing NADPH oxidase-mediated ROS generation and subsequent pathways leading to Ras activation, NF-κB promoter activity and VEGF gene expression. Our data indicate that suppression of p22phox and gp91phox expression would be a central mechanism for the anti-permeability effects of PEDF, thus providing a novel therapeutic potential of PEDF for the treatment of early diabetic retinopathy.

*In vitro*-modified AGEs were prepared by incubating BSA with glyceraldehyde for 1 week; this process produces relatively highly-modified proteins in comparison to those *in vivo*. However, it is unlikely that extensively-modified, unphysiologic AGEs that were formed under the *in vitro*-conditions may exert non-specific and toxic effects on vascular permeability for the following reasons: [1] We have previously found that immunological epitope of glyceraldehyde-modified AGEs was actually present in serum of diabetic patients and that the concentration (100
µg/ml) of in vitro-prepared AGEs used here were comparable with those of the in vivo diabetic situation (41,42) although we did not know the exact molecular pathway to form glyceraldehyde-derived AGEs in vivo; [2] we have shown in the present study that the AGE-rich serum fractions obtained from diabetic patients on hemodialysis (DM-AGEs) have the same biological effects as did the in vitro-prepared AGEs; DM-AGEs not only elicited retinal vascular hyperpermeability in rats, but also stimulated superoxide production in cultured ECs, a key downstream molecule of the AGE signaling to vasopermeability in vitro. These observations support the feasibility of using in vitro-prepared AGEs and suggest a causal role for endogenous AGEs in retinal vascular hyperpermeability in early diabetic retinopathy. In this study, Abs directed against in vitro-modified AGE that did not cross-react with well-defined AGE epitopes including pyrraline, pentosidine, carboxymethyllysine, and carboxyethyllysine, completely neutralized the AGE-induced ROS generation in ECs (Fig. 3C) (13,27). These findings suggest that structurally unidentified AGE epitope in the preparations of AGE-BSA or DM-AGEs may play a role in vascular permeability in diabetic retinopathy.

AGEs accumulate in various tissues in diabetes; most consistent AGE elevations are observed in retinal tissues rather than serum in diabetic retinopathy (48-50). In the present study, AGE administration increased retinal levels of an oxidative stress marker, 8-OHdG (Fig. 1H). Since oxidative stress generation could participate in formation of AGEs that are by themselves a source of the free radical superoxide generation (11,51), we can not exclude the possibility that intravenously injected AGEs may elicit retinal vascular permeability by enhancing AGE accumulation in the retinal vessels. Administration of AGEs to non-diabetic rats for 2 weeks was reported to stimulate AGE accumulation in the retinal vasculature, suggesting the possibility (50).

The extent of breakdown in the BRB observed here was relatively small, compared with that of Moore’s study where AGE-infusion caused about 400 % increase in Evan’s blue leakage (52). The difference of amounts and types of AGE-proteins injected into rats (ca. 4 mg/kg glyceraldehyde-derived AGEs vs 10 mg/kg glycolaldehyde-derived AGEs), animal species (SD rats vs C57/BL6 mice), or tracer (FITC-conjugated dextran vs Evans blue)
between our experiments and theirs could account for the discrepancies of the results.

The data presented here are highly dependent on the purity of PEDF proteins prepared. However, it is also unlikely that contaminants in the PEDF preparations could exert non-specific effects on retinal vessels because we have shown here that polyclonal Abs against PEDF reversed the anti-permeability effects of PEDF in DM-AGE-injected rats (Figure 1C and 1D). Furthermore, in the present study, we found that a single class of high affinity PEDF binding protein existed in cultured microvascular ECs (data not shown). These findings indicate that ECs could possess a system efficiently responding to PEDF, thus suggesting a possible involvement of EC surface receptor in the anti-permeability property of PEDF.

Ten nmol/L PEDF completely inhibited the ROS generation in AGE-exposed ECs, but it only partially blocked the Ras activation and subsequent signaling to vasopermeability. Furthermore, we have very recently found that PP1 analogue, which is an inhibitor of Src-family tyrosine kinases, inhibited the AGE-induced Ras activation, although it did not affect the ROS generation in ECs (unpublished data). These observations suggest that there exist at least two distinct signaling pathways to Ras activation in AGE-exposed ECs. One pathway is the NADPH oxidase-mediated ROS-dependent pathway which is suppressible by PEDF, and the other is a ROS-independent one which is mediated by Src-family kinase. There is a growing body of evidence that endothelial permeability as well as VEGF expression is regulated by Src kinase (53,54). These findings suggest the possible participation of Src-family kinase in the AGE-elicited vasopermeability.

Our present study has extended the previous findings showing that PEDF exerted antivasopermeability effects by counteracting the biological effects of VEGF (55). Thus the present study provides a novel beneficial aspect of PEDF on diabetic retinopathy; PEDF could prevent retinal vascular hyperpermeability, the characteristic changes of simple diabetic retinopathy by suppressing VEGF expression, thereby eliminating an important pathologic effect of AGE formation in vivo. Recently, PEDF was reported to inhibit the VEGF-induced angiogenesis by suppressing phosphorylation of VEGF receptor-1 (56). Therefore, PEDF could maintain cell-cell junctional integrity by blocking the VEGF-downstream signaling in AGE-exposed ECs. Since AGE administration
decreased retinal PEDF expression (Fig. 1F), pharmacological up-regulation or substitution of PEDF may offer a promising strategy for halting the development and progression of diabetic retinopathy.

**Perspectives**

In the present study, we did not examine the effects of PEDF on the AGE-induced angiogenesis. We are now studying whether PEDF treatment could have beneficial effects on proliferative diabetic retinopathy by suppressing the AGE-elicited VEGF expression.

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Footnotes

The abbreviations used are: AGEs, advanced glycation end products; VEGF, vascular endothelial growth factor; ROS, reactive oxygen species; PEDF, pigment epithelium-derived factor; ECs, endothelial cells; DPI, diphenylene iodonium; Abs, antibodies; BSA, bovine serum albumin; SD, Sprague-Dawley; BRB, blood retinal barrier; RT-PCR, reverse transcription–polymerase chain reactions; 8-OHdG, 8-hydroxydeoxyguanosine; TER, transendothelial electrical resistance; RAGE, receptor for AGEs; DHE, dihydroethidium; DN-Ras, dominant-negative human Ras mutant;
FIGURE LEGENDS

Figure 1. Anti-vasopermeability effects of PEDF in vivo. A, B, E-H: SD rats were injected intravenously with 1 mg AGE-BSA or non-glycated BSA in the presence or absence of 10 μg PEDF proteins or 10 μg Abs against rat VEGF every day for up to 10 days. C and D: SD rats were injected intravenously with 120 μL of serum containing AGE fractions derived from diabetic patients (DM-AGES) or healthy volunteers (Normal) in the presence or absence of 10 μg PEDF proteins or 77 μg polyclonal anti-human PEDF Abs every day for up to 5 days. Each group was composed of 4 SD rats. A and C: Typical microphotographs of the leakage of FITC-conjugated dextran from rat retinas. B and D: Quantification of BRB breakdown. E and G: Poly(A)+RNAs were isolated from enucleated eyes and then quantitative real-time RT-PCR was performed using Assay-on-Demand and TaqMan 5 fluorogenic nuclease chemistry. Relative gene expression levels of VEGF, p22phox and gp91phox are shown. F and H: Typical immunohistochemical staining of PEDF (F) and 8-OHdG (H) in rat retinas. GCL; ganglion cell layer, IPL; inner plexiform layer, INL; inner nuclear layer, OPL; outer plexiform layer, ONL, outer nuclear layer. #, P <0.05; *, P <0.01 compared to the value with AGEs or DM-AGEs alone.

Figure 2. Anti-vasopermeability effects of PEDF in vitro. A: ECs were treated with 100 μg/ml AGE-BSA or non-glycated BSA in the presence or absence of 10 nM PEDF for the indicated time periods, and then EC barrier function was assessed by measurement of TER. #, P <0.01 compared to the value with 100 μg/ml AGEs alone. *, P <0.01 compared to the value with 100 μg/ml non-glycated BSA alone. N=4 per group. Similar results were obtained in two independent experiments. B: ECs were treated with 100 μg/ml AGE-BSA or non-glycated BSA in the presence or absence of 10 nM PEDF for 4 h. Quantitative real-time RT-PCR was performed as described above. Relative
gene expression level of VEGF was shown. #, $P < 0.05$ compared to the value with 100 μg/ml AGEs alone. $N=4$ per group. Similar results were obtained in two independent experiments.

**Figure 3. Molecular mechanism for the suppression by PEDF of the AGE-induced VEGF gene overexpression in ECs.** A: Poly(A)$^+$RNAs were isolated from ECs treated with 100 μg/ml AGE-BSA or non-glycated BSA in the presence or absence of 10 nM PEDF for 4 h, and then analyzed by quantitative real-time RT-PCR. Panel shows the quantitative representation of p22phox and gp91phox gene induction. B, E and F: ECs were treated with 100 μg/ml AGE-BSA or non-glycated BSA in the presence or absence of 10 nM PEDF for 24 h, and then NADPH oxidase activity (B), Ras activation (E), and NF-κB promoter activity (F) were measured. C: ECs were treated with the indicated concentrations of AGE-BSA or non-glycated BSA in the presence or absence of 1 or 10 nM PEDF, 50 nM DPI, 10 μg/ml AGE-Abs, or 0.1 % anti-RAGE serum for 24 h, and then ROS were quantitatively analyzed. D: ECs were incubated with 2 % serum containing AGE fractions derived from diabetic patients (DM-AGEs) or healthy volunteers (Normal) in the presence or absence of 10 nM PEDF for 24 h. Superoxide generation was quantitatively analyzed. Typical microphotographs of the cells under a confocal microscopy are indicated. G: DN-Ras- or mock-transfected ECs were treated with 100 μg/ml AGE-BSA or non-glycated BSA for 24 h, and then NF-κB luciferase activity was measured. #, $P < 0.05$; *, $P < 0.01$ compared to the value with AGEs alone. $N=4-6$ per group. Similar results were obtained in two independent experiments.
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<tr>
<td><strong>Number of patients</strong></td>
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<tr>
<td><strong>Age (years)</strong></td>
<td>51.7 ± 8.6</td>
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<tr>
<td><strong>Duration of diabetes (years)</strong></td>
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<td><strong>HbA1c (%)</strong></td>
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<tr>
<td><strong>Number of patients with proliferative diabetic retinopathy</strong></td>
<td>11</td>
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Fig. 1 Yamagishi et al.
Fig. 2 Yamagishi et al.
Fig3 Yamagishi et al.
Pigment epithelium-derived factor (PEDF) inhibits advanced glycation end product-induced retinal vascular hyperpermeability by blocking reactive oxygen species-mediated vascular endothelial growth factor (VEGF) expression


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