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Short Communication

Pigment epithelium-derived factor prevents advanced glycation end products-induced monocyte chemoattractant protein-1 production in microvascular endothelial cells by suppressing intracellular reactive oxygen species generation

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Abstract

Aims/hypothesis. Monocytes and macrophages accumulate in the lesion of the diabetic retina, which are most likely involved in the progression of diabetic retinopathy. The levels of monocyte chemoattractant protein-1 (MCP-1) in vitreous fluids were associated with the severity of proliferative diabetic retinopathy. Recently, pigment epithelium-derived factor has been shown to be involved in the pathogenesis of proliferative diabetic retinopathy. However, a role of pigment epithelium-derived factor in monocyte recruitments in diabetic retinopathy remains to be elucidated. In this study, we investigated effects of purified pigment epithelium-derived factor on AGE-induced reactive oxygen species generation, *MCP-1* mRNA up-regulation and protein production in human cultured microvascular endothelial cells.
Methods. The intracellular formation of reactive oxygen species was measured using the fluorescent probe CM-H_2DCFDA. *MCP-1* gene expression was analysed in quantitative reverse transcription-polymerase chain reaction. Monocyte chemoattractant protein-1 production by microvascular endothelial cells was measured with an ELISA system.
Results. AGE increased intracellular reactive oxygen species generation in microvascular endothelial cells. Pigment epithelium-derived factor inhibited the AGE-induced reactive oxygen species generation in a dose-dependent manner. An anti-oxidant, *N*-acetylcysteine, or pigment epithelium-derived factor completely prevented the AGE-induced up-regulation of *MCP-1* mRNA contents as well as protein production in microvascular endothelial cells.
Conclusions/interpretations. Pigment epithelium-derived factor inhibits the AGE-induced reactive oxygen species generation and the subsequent increase in MCP-1 production in microvascular endothelial cells. Our study suggests that substitution of pigment epithelium-derived factor could prevent the progression of diabetic retinopathy by attenuating the deleterious effects of AGE. [Diabetologia (2003) 46:284–287]

Keywords AGE, PEDF, ROS, MCP-1, diabetic retinopathy.

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Abbreviations: MCP-1, monocyte chemoattractant protein-1; PDR, proliferative diabetic retinopathy; PEDF, pigment epithelium-derived factor; ROS, reactive oxygen species; EC, endothelial cells; NAC, N-acetylcysteine; glycer-AGE glyceraldehyde-derived AGE; glu-AGE, glucose-derived AGE; RT-PCR, reverse-transcription polymerase chain reaction.

There has been increasing interest in the role of inflammatory reaction in diabetic retinopathy [1]. Monocytes and macrophages accumulate in the lesion of the diabetic retina, being associated with the progression of diabetic retinopathy [1]. Furthermore, the levels of monocyte chemoattractant protein-1 (MCP-1) in vitreous fluids have been correlated with the severity of proliferative diabetic retinopathy (PDR) [2].

Pigment epithelium-derived factor (PEDF), a glycoprotein that belongs to the superfamily of serine protease inhibitors, was first purified from the conditioned media of human retinal pigment epithelial cells

as a factor with potent neuronal differentiating activity in human retinoblastoma cells [3]. Recently, PEDF has been shown to be a highly effective inhibitor of angiogenesis in cell culture and animal models; PEDF inhibited retinal-endothelial cell growth and migration and suppressed ischaemia-induced retinal neovascularization [4]. In addition, PEDF has been found in the vitreous and its levels were decreased in angiogenic eye diseases, suggesting that a loss of PEDF in the eye is functionally important in the pathogenesis of neovascularization in PDR [5]. However, a functional role of PEDF in monocyte recruitments in diabetic retinopathy has not been determined. We investigated the effects of purified PEDF proteins on AGE-induced reactive oxygen species (ROS) generation, *MCP-1* mRNA up-regulation and protein production in human cultured microvascular endothelial cells (EC).

Materials and methods

Materials. BSA (fraction V) and *N*-acetylcysteine (NAC) were purchased from Sigma (St. Louis, Mo., USA). *D*-glyceraldehyde was from Nakalai Tesque (Kyoto, Japan). *D*-glucose was purchased from Wako Pure Chemical Industries (Osaka, Japan). [γ-^{32}P]ATP was from Amersham Pharmacia Biotech (Buckinghamshire, United Kingdom). MCP-1 ELISA systems were from R&D systems (Minneapolis, Minn., USA).

Construction of PEDF expression vector PEDF. cDNA was originally cloned from human placenta cDNA library (Clontech, Palo Alto, Calif., USA), and inserted into the mammalian expression vector pBK-CMV (Stratagene, La Jolla, Calif., USA).

Purification of PEDF proteins. 293T cells (ATCC, Rockville, Md., USA) were transfected with hexahistidine-tagged PEDF expression vector using a FuGENE 6 transfection reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacture's instructions. Then PEDF proteins were purified from conditioned media by a Ni-NTA spin kit (Qiagen GmbH, Hilden, Germany) according to the manufacture's instructions. SDS-PAGE analysis of purified PEDF proteins showed a single band with a molecular weight of about 50 M_r, which showed positive reactivity with monoclonal antibody against human PEDF.

Preparation of AGE-proteins. AGE-BSA was prepared by incubating BSA with *D*-glyceraldehyde (glycer-AGE) or *D*-glucose (glu-AGE) as described previously [6]. Non-glycated BSA was incubated in the same conditions except for the absence of reducing sugars.

Cells. The human adult skin microvascular EC were cultured as described previously [6].

Intracellular ROS. Endothelial cells were incubated with 100 μg/ml of glycer-AGE-BSA, glu-AGE-BSA or non-glycated BSA in the presence or absence of various concentrations of PEDF proteins for 1 day. Then the intracellular formation of ROS was measured using the fluorescent probe CM-H_2DCFDA (Molecular Probes, Eugene, Ore., USA) [7].

Confocal microscopy. Endothelial cells were treated with 100 μg/ml glycer-AGE-BSA or non-glycated BSA in the presence or absence of 10 nmol/l PEDF proteins for 1 day, and then incubated with CM-H_2DCFDA for 7 min. The cells were immediately imaged under a laser-scanning confocal microscope, and the fluorescence was measured in three randomly selected fields.

Primers and probes. Sequences of the sense and antisense primers and the internal probe were 5′-AAC TGA AGC TCG CAC TCT CG-3′, 5′-TCA GCA CAG ATC TCC TTG GC-3′, and 5′-GTG ATC TTC AAG ACC ATT GT-3′ for detecting human *MCP-1* mRNA. The primers and probe used for human β-*actin* mRNA detection were the same as in a previous study [8].

Quantitative reverse transcription-polymerase chain reaction (RT-PCR). Poly(A)$^+$RNAs were isolated from EC treated with 100 μg/ml of AGE-BSA or non-glycated BSA in the presence or absence of 10 nmol/l PEDF proteins or 1 mmol/l NAC for 4 h, and then analysed by RT-PCR as described previously [8]. The amounts of poly(A)$^+$RNA templates (30 ng) and cycle numbers (30 cycles) for amplification were chosen in quantitative ranges, where reactions proceeded linearly, which had been determined by plotting signal intensities as functions of the template amounts and cycle numbers [9].

Measurement of MCP-1. Endothelial cells were incubated with 100 μg/ml of glycer-AGE-BSA or non-glycated BSA in the presence or absence of 10 nmol/l PEDF proteins or 1 mmol/l NAC for 1 day. Then MCP-1 proteins released into media were measured with ELISA systems according to the manufacture's instructions [7].

Statistical analysis. All values were presented as means ± SE. Statistical significance was evaluated using the Student's *t* test for paired comparison; a *p* value of less than 0.05 was considered significant.

Results

PEDF inhibits AGE-induced ROS generation in EC. First we investigated whether glycer-AGE treatment can stimulate intracellular ROS generation in cultured microvascular EC. Glycer-AGE increased intracellular ROS generation to about 1.4-fold (Fig. 1A). Purified PEDF proteins inhibited the glycer-AGE-induced increase in ROS generation in a dose-dependent manner; at the concentration of 10 nmol/l, PEDF proteins completely prevented the glycer-AGE-induced ROS generation in EC. We obtained the same results with glu-AGE; glu-AGE-stimulated ROS generation in EC, and 10 nmol/l PEDF proteins completely blocked the ROS generation elicited by glu-AGE. PEDF proteins of 10 nmol/l alone did not affect ROS generation in EC. Next, we investigated whether PEDF proteins could actually decrease fluorescence intensities in glycer-AGE exposed EC using a laser-scanning confocal microscope. Glycer-AGE increased fluorescence intensities in EC (Fig. 1B). Treatments with PEDF

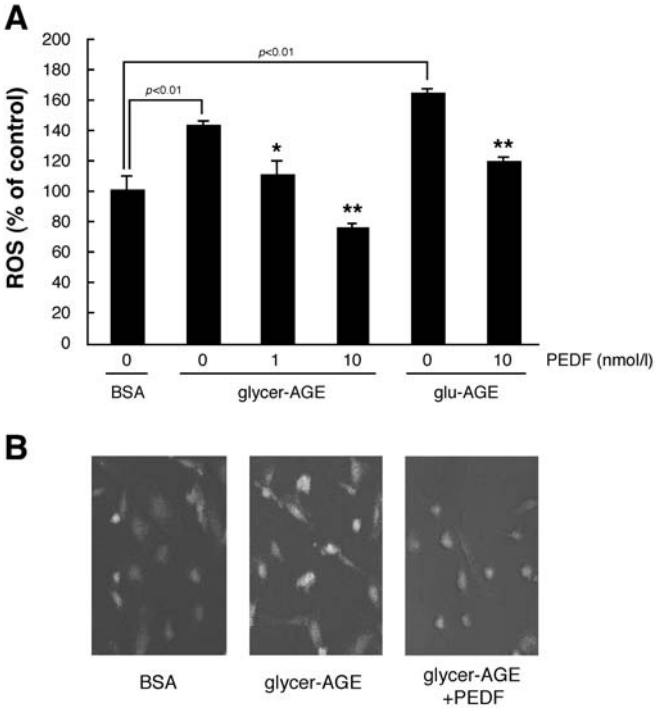

Fig. 1A, B. Effects of PEDF proteins on intracellular ROS generation in microvascular EC. (**A**) EC were incubated with 100 μg/ml of glycer-AGE-BSA, 100 μg/ml of glu-AGE-BSA or non-glycated BSA in the presence or absence of the indicated concentrations of PEDF proteins for 24 h, and then ROS were quantitatively analysed. **p*<0.05; ***p*<0.01 compared to the value of the control with AGE alone. (**B**) Typical microphotographs of cells under confocal microscopy. EC were incubated with 100 μg/ml of glycer-AGE-BSA or non-glycated BSA in the presence or absence of 10 nmol/l PEDF proteins for 24 h, and then imaged by a laser-scanning confocal microscope

proteins completely blocked the glycer-AGE-induced increase in fluorescence intensities in EC.

PEDF inhibits AGE-induced MCP-1 gene expression in EC. Because the *MCP-1* gene is known to be induced by ROS [7], we studied whether glycer-AGE can up-regulate *MCP-1* mRNA contents in EC. Glycer-AGE increased *MCP-1* mRNA contents to about 1.7-fold (Fig. 2A, B) PEDF or NAC treatment completely prevented the glycer-AGE-induced up-regulation of *MCP-1* mRNA contents in EC. We found that glu-AGE-elicited *MCP-1* mRNA induction was also blocked by PEDF treatments.

PEDF inhibits glycer-AGE-induced MCP-1 overproduction by EC. We investigated whether glycer-AGE can actually stimulate MCP-1 production by microvascular EC. Glycer-AGE increased MCP-1 production by EC to about 1.3-fold (Fig. 2C). PEDF or NAC treatment completely prevented the glycer-AGE-induced MCP-1 overproduction by EC.

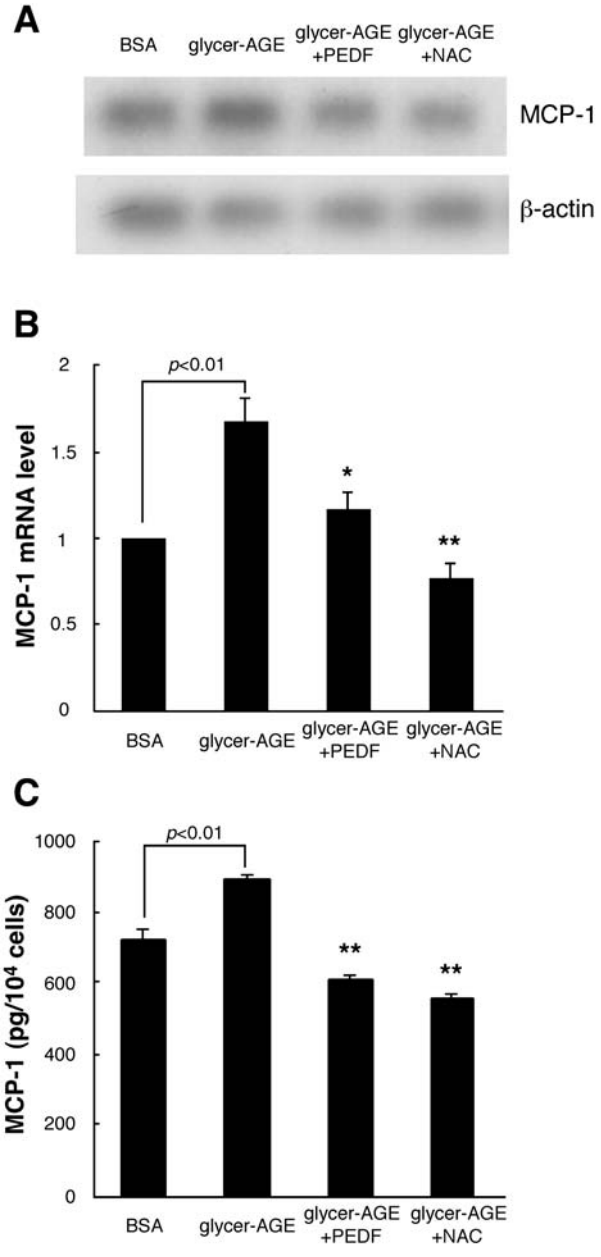

Fig. 2A–C. Effects of PEDF proteins on *MCP-1* gene and mRNA expression in microvascular EC. (**A**) Poly(A)$^+$RNAs were isolated from EC treated with 100 μg/ml of glycer-AGE-BSA or non-glycated BSA in the presence or absence of 10 nmol/l PEDF proteins or 1 mmol/l NAC for 4 h, and then analysed by RT-PCR. Each lower panel shows the expression of *β-actin* genes. PCR amplification for *β-actin* mRNA was performed for 22 cycles. (**B**) Quantitative representation of *MCP-1* gene induction. Data were normalized by the intensity of *β-actin* mRNA-derived signals and related to the value with non-glycated BSA alone. Similar results were obtained in two independent experiments. **p* <0.05; ***p* <0.01 compared to the value of the control with glycer-AGE alone. (**C**) EC were incubated with 100 μg/ml of glycer-AGE-BSA or non-glycated BSA in the presence or absence of 10 nmol/l PEDF proteins or 1 mmol/l NAC for 1 day. Then MCP-1 proteins released into media were measured with an ELISA kit. ***p*<0.01 compared to the value of the control with glycer-AGE alone

Discussion

Our study shows that PEDF proteins inhibited the AGE-induced ROS generation, *MCP-1* mRNA up-regulation and protein production in human cultured microvascular EC. The AGE-elicited ROS generation induced *MCP-1* gene and mRNA induction in EC due to the following: (i) First, AGE increased intracellular ROS generation in microvascular EC; (ii) an anti-oxidant, NAC, completely prevented the AGE-induced *MCP-1* mRNA up-regulation and overproduction in EC. These results suggest that PEDF inhibited MCP-1 overproduction through its anti-oxidative properties. We found no additive effects with NAC on *MCP-1* gene expression , further supporting a causal relation between PEDF actions and its anti-oxidative activity.There has been increasing interest in the role of inflammatory reaction and immune phenomenon in the pathogenesis of diabetic retinopathy. Leukocyte adhesion to diabetic retinal vasculatures has been considered as a critical early event in diabetic retinopathy [10], and monocytes and macrophages accumulate in the lesion of the diabetic retina, being associated with the progression of diabetic retinopathy [1]. Furthermore, the levels of MCP-1 in vitreous fluids were correlated with the severity of PDR [2]. These observations suggest that PEDF could inhibit the recruitment of monocytes into the eyes, thus preventing the development of PDR.Vitreal PEDF was recently found to be decreased in angiogenic eye diseases, suggesting that a loss of PEDF in the eye is functionally important in the pathogenesis of neovascularization in PDR [5]. Our present study provided another harmful aspect of PEDF loss in diabetic retinopathy; loss of PEDF in the eye could potentiate the deleterious effects of AGE in vivo. Since there is a growing body of evidence that formation and accumulation of AGE in the retina play a pivotal role in the pathogenesis of diabetic retinopathy [6, 8, 9], our observations suggest that substitution of PEDF could be a promising strategy for treatment of patients with PDR.

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