Advanced glycosylation end products stimulate the growth but inhibit the prostacyclin-producing ability of endothelial cells through interactions with their receptors

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Abstract The influence of advanced glycosylation end products (AGE) on endothelial cells was investigated. When human umbilical endothelial cells were cultured with AGE-bovine serum albumin, viable cell number as well as DNA synthesis was significantly stimulated, whereas prostacyclin production by the endothelial cells was decreased. Antisense oligodeoxyribonucleotides against mRNA coding for AGE receptor were found to reverse both the AGE-induced growth stimulation and the inhibition of prostacyclin production in endothelial cells. These results thus suggest that AGE ligand–receptor interactions in endothelial cells can promote angiogenesis and thrombogenesis, leading to the development of diabetic vascular complications.

Key words: Advanced glycosylation end products (AGE); Receptor for AGE (RAGE); Antisense oligodeoxyribonucleotide; Endothelial cell; Pericyte; Diabetic angiopathy

1. Introduction

Glucose and other reducing sugars can react non-enzymatically with the amino groups of proteins to form reversible Schiff bases and then Amadori products. These early glycosylation products undergo further reactions and rearrangements to become irreversible crossed-linked, fluorescent protein derivatives termed advanced glycosylation end products (AGE). AGE are known to accumulate in various tissues of diabetic subjects, and are implicated in the development of diabetic complications [1].

We have shown recently that AGE can retard the growth of vascular pericytes and exhibit an immediate toxicity to these microvascular constituents through interactions with their receptors, and proposed a novel mechanism for pericyte loss, the earliest histopathological hallmark in diabetic retinopathy [2,3].

In this paper, we describe the effects of AGE on the growth and prostacyclin-producing ability of endothelial cells. A possible participation of the endothelial AGE ligand–receptor system in the development and progression of diabetic angiopathies is discussed.

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Abbreviations: AGE, advanced glycosylation end products; BSA, bovine serum albumin; PBS, phosphate-buffered saline; FBS, fetal bovine serum; 6-keto-$PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$; RAGE, receptor for AGE; RT-PCR, reverse transcription-polymerase chain reaction.

2. Materials and methods

2.1. Preparation of AGE

AGE were prepared by incubating bovine serum albumin (BSA) (fraction V, fatty acid-free, low endotoxin; Boehringer Mannheim GmbH, Germany) with 500 mM glucose in phosphate-buffered saline (PBS) (10 mM, pH 7.4) at 37°C for 6 weeks under sterile conditions [2]. Unincorporated sugars were removed by dialysis against PBS. AGE-BSA were purified by heparin-Sepharose CL-4B (Pharmacia LKB, Uppsala, Sweden) column chromatography; separation of AGE-BSA from non-glycated BSA was confirmed by SDS-PAGE. The concentration of AGE-BSA was determined by the method of Bradford [4].

2.2. Cells

Endothelial cells were obtained from human umbilical vein and maintained in Roswell Park Memorial Institute tissue culture medium 1640/Medium 199 (1/1) (Life Technologies, Grand Island, NY, USA) supplemented with 15% fetal bovine serum (FBS) (ICN Biomedicals Inc., Costa Mesa, CA, USA), 100 μg/ml endothelial cell growth supplement (Collaborative Research, Bedford, USA) and 25 μg/ml heparin [5].

2.3. Determination of viable cell number

Endothelial cells cultured for various time periods in the presence or absence of AGE were dislodged with trypsin, and counted by the dye exclusion method [6].

2.4. Measurement of [^{3}H]thymidine incorporation

[^{3}H]thymidine incorporation was determined as described previously [7]. Briefly, endothelial cells were seeded at a density of 2×10^4 cells per well of a Costar 24-well cluster dish and kept at 37°C overnight. After cell attachment, AGE were added to the medium, and cells were incubated for 24 h. Then [^{3}H]thymidine was added to a final concentration of 1 μCi/ml, and cells were further incubated for 4 h. After incubation, cells were fixed with ice-cold 10% (w/v) trichloroacetic acid for 20 min, and the resultant acid-insoluble materials were processed for liquid scintillation counting.

2.5. Measurement of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$)

6-keto-$PGF_{1\alpha}$ released into media was measured with an Amersham enzyme immunoassay system according to the supplier's recommendation.

2.6. Assay with antisense oligodeoxyribonucleotides

A phosphorothioate antisense complement of human receptor for AGE (RAGE) mRNA, and the corresponding sense oligodeoxyribonucleotide, were synthesized and purified as described previously [2]. Sequences of antisense and sense oligonucleotides were 5′-CAACTG-CTGTTCCGGCT-3′ and 5′-AGCCGGAACAGCAGTTG-3′, respectively, which corresponded to nucleotides 6–22 of human cDNA [8]. The oligonucleotides were added to the medium, with 0.05 mg/ml AGE-BSA, in which endothelial cells were grown. After 4 h, [^{3}H]thymidine incorporation and 6-keto-$PGF_{1\alpha}$ production were assayed.

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2.7. Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNAs isolated from endothelial cells were analyzed by RT-PCR as described previously, using primers specific to the human RAGE mRNA [2]. Primer sequences for detecting β-actin mRNA were as given in [7]. Southern blot analysis of the RT-PCR products was performed with respective ^{32}P-end labelled probes [2,7]. Template amounts and cycle numbers for amplification were chosen in quantitative ranges, which were determined by plotting signal intensities as functions of template amounts and of cycle numbers [7]. Signal intensities of hybridized bands were measured by a Fujix BAS 1000 image analyzer (Fuji Photo Film Co. Ltd., Hamamatsu, Japan).

3. Results

3.1. Effects of AGE on the growth of endothelial cells

Endothelial cells from human umbilical vein were cultured in the presence or absence of AGE-BSA, and the viable cell number was determined at days 1, 2 and 3 after AGE addition. As shown in Fig. 1A, the growth curve was found to be shifted upwards by the treatment with AGE-BSA. At 0.1 mg/ml, the growth rate was about 2-fold higher than that of the untreated cells; the AGE-induced increase was statistically significant at days 2 and 3 at every concentration tested. AGE also significantly stimulated DNA synthesis in endothelial cells (Fig. 1B). Non-glycated BSA induced no change in either the cell number or DNA synthesis.

3.2. Effects of AGE on the prostacyclin production

We next tested whether AGE affect the ability of endothelial cells to produce prostacyclin, the key substance endowing the endothelium with anti-thrombogenic activities. When assayed for its stable metabolite, 6-keto-PGF$_{1\alpha}$, prostacyclin production by endothelial cells was significantly lowered by AGE-BSA (Fig. 2). At 0.05 mg/ml, the amount of 6-keto-PGF$_{1\alpha}$ released by endothelial cells was decreased to about half of the control value without AGE-BSA.

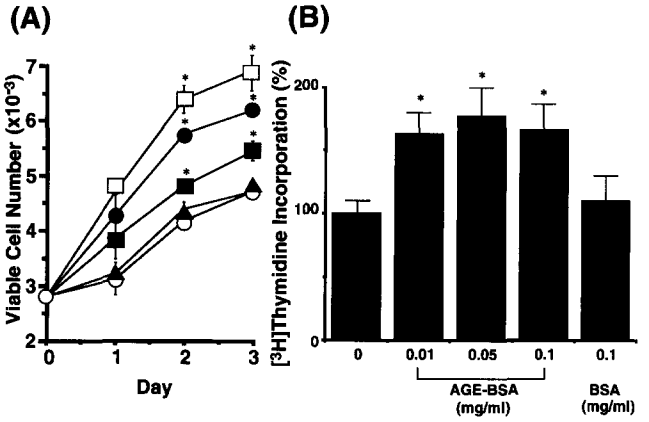

Fig. 1. Effects of AGE-BSA on viable cell number (A), and on DNA synthesis (B) in human umbilical vein endothelial cells. (A) Endothelial cells were grown in the presence of 0.01 (■), 0.05 (●) or 0.1 (□) mg/ml AGE-BSA or 0.1 mg/ml non-glycated BSA (▲), or in the absence of BSA (○). Culture period after the addition of AGE-BSA is indicated on the abscissa, and the viable cell number on the ordinate. Each point represents the mean ± S.E.M. of 3 replicate experiments. (B) Endothelial cells were treated with 0.01, 0.05 or 0.1 mg/ml AGE-BSA, or 0.1 mg/ml non-glycated BSA, or without them for 24 h. [^{3}H]thymidine incorporation was determined as described under section 2 and related to the value for the control with no additives. Each column represents the mean ± S.E.M. of 3 replicate experiments. *$P < 0.05$, compared to both the control value without additives and that with non-glycated BSA (Student's *t*-test).

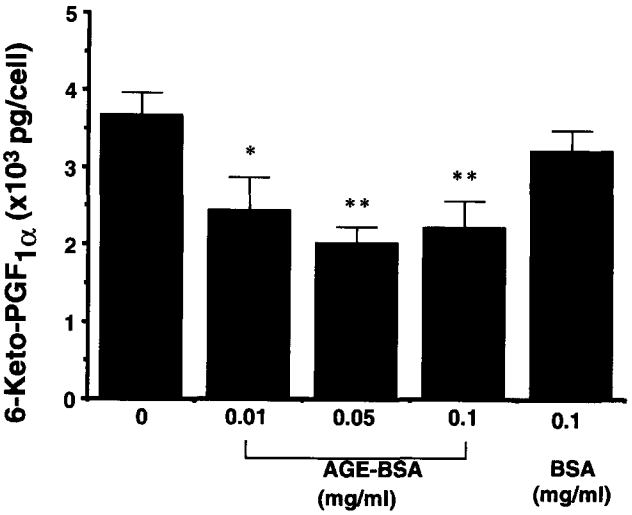

Fig. 2. Effects of AGE-BSA on 6-keto-PGF$_{1\alpha}$ production in human umbilical vein endothelial cells. Amounts of 6-keto-PGF$_{1\alpha}$ released by endothelial cells are indicated on the ordinate. Endothelial cells were treated with 0.01, 0.05 or 0.1 mg/ml AGE-BSA, or 0.1 mg/ml non-glycated BSA, or without them for 2.5 days. Each column represents the mean ± S.E.M. of 9 replicate experiments. * and **, $P < 0.05$ and $P < 0.01$, respectively, compared to both the control value without additives and that with non-glycated BSA (Student's *t*-test).

3.3. Reversal of the AGE actions on endothelial cells by antisense oligonucleotides against RAGE mRNA

As we and others have shown previously, endothelial cells possess a cell surface RAGE [2,8]. We then examined its functional role in the AGE effects on endothelial cells, by manipulating the expression of RAGE gene with antisense DNA. Septadecamer antisense oligodeoxyribonucleotides complementary to human RAGE mRNA, and the corresponding sense control, were administered to culture media, in which endothelial cells were grown. As shown in Fig. 3A and B, the antisense oligomers were found to significantly diminish both the AGE-induced increase in DNA synthesis and the inhibition of prostacyclin production in endothelial cells; at 10 μM, complete reversals were obtained. Sense oligomers did not affect the AGE actions.

Evidence that the antisense oligomers did inhibit the expression of the RAGE gene was obtained. Quantitative RT-PCR analysis confirmed the presence of RAGE mRNA in endothelial cells, and revealed that the antisense DNA could reduce the content of the target mRNA to about one-half of that of untreated control when standardized with the signal intensity of β-actin mRNA (Fig. 4).

4. Discussion

The present study demonstrated for the first time that AGE stimulate the growth but inhibit the prostacyclin-producing ability of human endothelial cells. The maximal effect of AGE was noted at 0.05 rather than 0.1 mg/ml in both DNA synthesis and prostacyclin production, but the dose–response relationship in the two indices was a striking mirror-image (see Fig. 1B and 2). These effects could be reversed by antisense DNA against RAGE mRNA, indicating that these AGE actions on endothelial cells are mediated by the ligand–receptor interaction. The antisense DNA-induced de-

crease in RAGE mRNA (Fig. 4) was probably through RNase H- and RNase H-like activity-driven degradation [9]. The inhibition of binding and transition of ribosomes on target mRNA [10] would seem to help account for the antisense effects. Although the AGE-induced changes appeared to be modest, chronic exposure to AGE accumulated during prolonged hyperglycemic states may give rise to local deterioration in the growth or function of the endothelium. Tezuka et al. reported that similar concentrations of AGE stimulated the migration and tube formation of human umbilical vein endothelial cells but did not affect their proliferation [11]. Although no explanation as to why the endothelial cell growth under AGE differed between the two studies is available, the differences in the type of growth supplements employed or in the AGE preparations may be responsible.

The response of pericytes to AGE contrasts with that of endothelial cells. As we have shown previously [2], the growth of the former is decreased by the AGE ligand–receptor interaction. That AGE can differentially influence the growth of these two microvascular constituents would seem to be of significance. First, an in vivo sequela would be angiogenesis in either case. Because pericytes have been shown to halt endothelial cell growth [5,12], the AGE-induced decrease in the pericyte number would relieve this restriction and allow the neighboring endothelial cells to grow unchecked. Second, the post-AGE receptor signalling events may diverge even between vascular endothelial cells and pericytes. How the ligand binding leads to the stimulation or to the repression of replication in the respective cell type is an important issue to be clarified.

Thrombogenesis would be another sequela of the AGE action on either endothelial cells or pericytes. Endothelial cell production of prostacyclin was inhibited by AGE (Fig. 2);

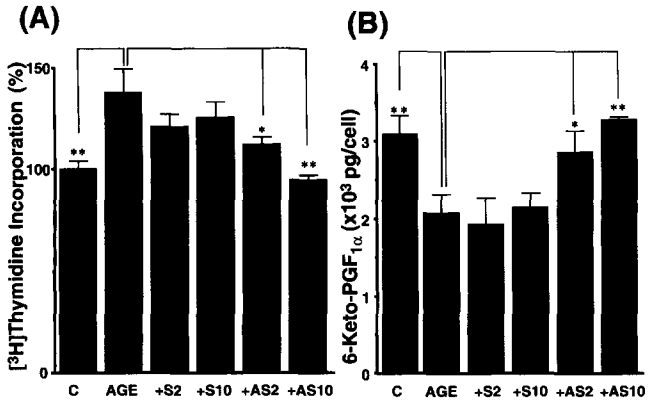

Fig. 3. Effects of antisense oligonucleotides on the AGE-induced stimulation of DNA synthesis (A) and the inhibition of prostacyclin production (B) in endothelial cells. Endothelial cells were cultured with 0.05 mg/ml AGE in the presence or absence of either 2 μM or 10 μM oligomer or with neither AGE-BSA nor oligomers. (A) The percentage of [^{3}H]thymidine incorporation is indicated on the ordinate. (B) The amount of 6-keto-PGF$_{1\alpha}$ released by endothelial cells is indicated on the ordinate. Each column represents the mean values of 6 (A) or 4 (B) replicate experiments. Bars show S.E.M. * and **, $P < 0.1$ and $P < 0.05$, respectively, compared to the values obtained in the presence of AGE alone (Student's t-test). C, control without additives; AGE, 0.05 mg/ml AGE-BSA; +S2, 0.05 mg/ml AGE-BSA plus 2 μM sense oligomers; +S10, 0.05 mg/ml AGE-BSA plus 10 μM sense oligomers; +AS2, 0.05 mg/ml AGE-BSA plus 2 μM antisense oligomers; +AS10, 0.05 mg/ml AGE-BSA plus 10 μM antisense oligomers.

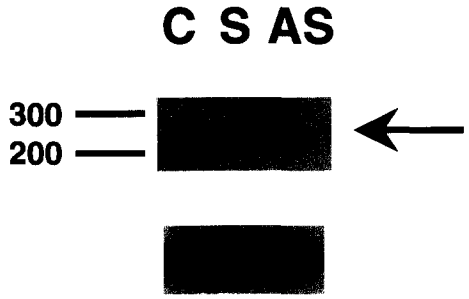

Fig. 4. Quantitative RT-PCR analysis of RAGE mRNA. Total RNAs from endothelial cells cultured with or without oligonucleotides for 8 h, a time period equivalent to that for the determination of DNA synthesis, were analyzed by RT-PCR; 100 ng of each RNA template was reverse-transcribed and amplified at 35 cycles where the reactions proceeded exponentially. Arrow indicates the position of the RT-PCR products (286 base pairs). C, control without oligomers; S, 10 μM sense oligomers; AS, 10 μM antisense oligomers. Size markers (base pairs) are shown on the left. Lower panel shows signals derived from β-actin mRNA. RT for the latter was performed with 100 ng of RNA templates, and the PCR amplification was at 30 cycles.

and, because pericyte–endothelial cell interactions are required to preserve the endothelial ability to synthesize prostacyclin [5], the AGE-induced growth retardation as well as their immediate toxicity in pericytes [2] should indirectly result in a focal decrease in the production of this anti-thrombogenic prostanoid. In addition, Esposito et al. [13] have shown that AGE can suppress the endothelial cell surface activity of the anti-coagulant factor thrombomodulin, and can induce the expression of procoagulant cofactor tissue factor, the consequences of both of which are again thrombogenesis.

In conclusion, the available evidence suggests that AGE can drive endothelial cells to proliferate and to become thrombogenic on one hand, and can damage pericytes on the other, leading to their dropout, which would in turn impair pericyte–endothelial interactions, further exacerbating endothelial dysfunction. AGE may thus promote angiogenesis and thrombogenesis, thereby playing an active part in the development and progression of diabetic angiopathy.

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