Advanced Glycation End Products-driven Angiogenesis in Vitro

INDUCTION OF THE GROWTH AND TUBE FORMATION OF HUMAN MICROVASCULAR ENDOTHELIAL CELLS THROUGH AUTOCRINE VASCULAR ENDOTHELIAL GROWTH FACTOR*

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Sho-ichi Yamagishi‡, Hideto Yonekura‡, Yasuhiko Yamamoto‡, Kenji Katsuno§, Fumiyasu Sato§, Izumi Mita¶, Hisayoshi Ooka¶, Noboru Satozawa†, Takuhisa Kawakami‡, Motohiro Nomura‡, and Hiroshi Yamamoto†***

From the ‡Department of Biochemistry, Kanazawa University School of Medicine, Kanazawa 920, Japan, the §Discovery Research Laboratory 3, Kissei Pharmaceutical Co. Ltd., Hitotsubashi 388-83, Japan, the ¶Institute of Biological Science, Mitsui Pharmaceuticals Inc, Mobara 297, Japan, and the ¶Life Science Laboratory, Mitsui Toatsu Chemicals Inc., Mobara 297, Japan

This study was undertaken to determine whether and how advanced glycation end products (AGE), senescent macroproteins accumulated in various tissues under hyperglycemic states, cause angiogenesis, the principal vascular derangement in diabetic microangiopathy. We first prepared AGE-bovine serum albumin (BSA) and anti-AGE antiserum using AGE-RNase A. Then AGE-BSA was administered to human skin microvascular endothelial cells in culture, and their growth was examined. The AGE-BSA, but not nonglycated BSA, was found to induce a statistically significant increase in the number of viable endothelial cells as well as their synthesis of DNA. The increase in DNA synthesis by AGE-BSA was abolished by anti-AGE antibodies. AGE-BSA also stimulated the tube formation of endothelial cells on Matrigel. We obtained the following evidence that it is vascular endothelial growth factor (VEGF) that mainly mediates the angiogenic activities of AGE. (1) Quantitative reverse transcription-polymerase chain reaction analysis of poly(A)+ RNA from microvascular endothelial cells revealed that AGE-BSA up-regulated the levels of mRNAs for the secretory forms of VEGF in time- and dose-dependent manners, while endothelial cell expression of the genes encoding the two VEGF receptors, kinase insert domain-containing receptor and fms-like tyrosine kinase 1, remained unchanged by the AGE treatment. Immunoprecipitation analysis revealed that AGE-BSA did increase de novo synthesis of VEGF. (2) Monoclonal antibody against human VEGF completely neutralized both the AGE-induced DNA synthesis and tube formation of the endothelial cells. The results suggest that AGE can elicit angiogenesis through the induction of autocrine vascular VEGF, thereby playing an active part in the development and progression of diabetic microangiopathies.

Glucose and other reducing sugars can react nonenzymatically with the amino groups of proteins to form reversible Schiff bases and, then, Amadori products. These early glycation products undergo further complex reactions such as rearrangement, dehydration, and condensation to become irreversibly cross-linked, heterogeneous fluorescent derivatives termed advanced glycation end products (AGE)1. The formation and accumulation of AGE in various tissues have been known to progress during normal aging and at an extremely accelerated rate in diabetes mellitus. This has been implicated in the development of diabetic micro- and macro-vascular complications (1), which may account for the disabilities and high mortality rate in patients with this disease (2).

Microvessels are composed of only two types of cells, endothelial cells and pericytes, and have been known to show both functional and structural abnormalities during prolonged diabetic exposure, resulting in the deleterious effects on the organs that they supply (3–5). Using pericyte-endothelial cell co-culture systems, we have shown previously that pericytes can not only regulate the growth but also preserve the prostacyclin-producing ability and protect against lipid peroxide-induced injury of endothelial cells (6). This has provided a basis for understanding how diabetic retinopathy develops consequent to “pericyte loss,” the earliest histopathological hallmark in diabetic retinopathy (5, 7).

Recently, we have found that AGE exert a growth inhibitory effect and a cell type-specific immediate toxicity on pericytes through interactions with their receptor for AGE (RAGE), a cell surface receptor belonging to the immunoglobulin superfamily (8), and have proposed a novel mechanism for pericyte loss (9). The AGE-induced, RAGE-mediated decrease in pericyte number would then indirectly cause angiogenesis (6, 9).

In the present study, we investigated the effects of AGE on the growth and tube formation of human skin microvascular endothelial cells, the key steps of angiogenesis. We demonstrate that AGE exert angiogenic activities directly on microvascular endothelial cells and that autocrine vascular endothelial growth factor (VEGF) is the major mediator of the AGE-driven angiogenesis.

EXPERIMENTAL PROCEDURES

Materials—Bovine serum albumin (BSA) was purchased from Boehringer Mannheim GmbH (Mannheim, Germany). Bovine pancreatic RNase A, bovine hemoglobin (Hb), N⁴-tosyl-lysine methyl ester and phenylmethylsulfonyl fluoride were from Sigma. Heparin-Sepharose

1 The abbreviations used are: AGE, advanced glycation end products; RAGE, receptor for AGE; VEGF, vascular endothelial growth factor; BSA, bovine serum albumin; Hb, hemoglobin; HRP, horseradish peroxidase; CM-TsLME, N⁴-tosyl-N⁴-carboxymethyllysine methyl ester; CM-BSA, N⁴-carboxymethylated BSA; ELISA, enzyme-linked immunosorbent assay; RT-PCR, reverse transcription-polymerase chain reaction; F1t, fms-like tyrosine kinase 1; KDR, kinase insert domain-containing receptor; MoAb, monoclonal antibody; bp, base pairs.

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** To whom correspondence and requests for reprints should be addressed. Tel.: 81-76-265-2180; Fax: 81-76-234-4226.
CL-4B was from Pharmacia LKB (Uppsala, Sweden). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was from BioMakor (Rehovot, Israel). [3H]Thymidine and [γ-32P]ATP were from DuPont NEN. Reverse transcriptase and T4 polynucleotide kinase were from Takara (Kyoto, Japan). Hybond-N+ nylon membrane was from Amer sham Corp. (Buckinghamshire, United Kingdom). Matrigel was from Collaborative Research (Bedford, MA).

Preparation of AGE-Proteins and Amadori Compounds—BSA (fraction V, fatty acid-free, free endotoxin) was incubated with 0.5% glucose at 37°C for 6 weeks under sterile conditions in the presence of 1.5 mM phenylmethylsulfonyl fluoride, 0.5 mM EDTA, 100 units/ml penicillin, and 40 μg/ml gentamycin (9–11). After unincorporated sugars were removed by dialysis against phosphate-buffered saline, glucose-modified high molecular weight materials were purified by heparin-Sepharose CL-4B column chromatography and used as AGE-BSA. Control nonglycated BSA was incubated in the same conditions except for the absence of glucose. The concentration of AGE-BSA was determined by the method of Bradford (12). AGE-RNase A and AGE-Hb were prepared according to the method of Makita et al. (13). For reducing AGE-Hb, NaBH₄, was employed as described by Horiuchi et al. (14). 1-Deoxy-1-propylaminod-ß-fructose, an Amadori compound, was synthesized by the method of Michael and Hagemann (15). N-ß-Toyl-N′-carboxymethyl-lysine methyl ester (CM-TsLME) was synthesized from N-ß-toxy-lysine methyl ester by the method of Ahmed et al. (16) with minor modification. N-Carboxymethylated BSA (CM-BSA) was prepared according to the method of Reddy et al. (17).

Preparation of Anti-AGE-RNase A Antiserum—1 mg of AGE-RNase A was emulsified in 50% Freund complete adjuvant and injected intradermally into rabbits. Two weeks later, a booster with the same amount of AGE-RNase A was administered, followed by nine additional booster injections, with one given every 2–3 weeks. Ten days after the final injection, the antiserum was obtained.

Enzyme-linked Immunosorbent Assay (ELISA)—In the noncompetitive ELISA system, wells of 96-well microtiter plates were coated with increasing amounts of Hb, AGE-Hb, and reduced AGE-Hb. After washing and blocking, the wells were incubated with 100 μl of anti-AGE antiserum (1:4000) for 2 h and then with 100 μl of HRP-conjugated goat anti-rabbit IgG for 30 min. Finally, 100 μl of substrate tetramethylbenzidine solution was added into each well. After 10–15 min, the absorbance at 450 nm was measured. In the competitive ELISA system, procedures similar to the noncompetitive ELISA were used except for the following two points. Wells were first coated with reduced 100 ng/ml AGE-Hb solution as absorbent antigens, and then 50 μl of test samples were added as a competitor together with 50 μl of anti-AGE antiserum (1:4000) into each well.

Cells—Endothelial cells from human skin microvessels were maintained in E-BM 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 instructions (Cletonics Corp., San Diego, CA). Cells at 5–10 passages were used for the experiments. AGE treatment was carried out in a medium lacking epidermal growth factor and hydrocortisone.

Measurement of Cell Growth—Endothelial cells cultured for various time periods in the presence or absence of AGE-BSA were dislodged with trypsin, and counted by the dye exclusion method (18). [3H]Thymidine incorporation was determined as described previously (19). For determining the effects of anti-AGE antiserum on endothelial cell growth, the antiserum was added to the medium at 1/1000 (v/v) together with or without 50 μg/ml AGE-BSA, after which cells were incubated for 24 h and [3H]thymidine incorporation was measured.

Primers and Probes—Oligonucleotide primers and probes for quantitative reverse transcription-polymerase chain reactions (RT-PCR) were synthesized by a Perkin-Elmer 392 DNA synthesizer (Foster City, CA) and purified as described previously (20). Primer sequences and internal oligonucleotide probes for detecting VEGF, fms-like tyrosine kinase 1 (flt 1), kinase insert domain-containing receptor (kdr), and β-actin mRNA were the same as described in Ref. 20.

Quantitative RT-PCR—Poly(A)+ RNAs were isolated (21) from cells treated with or without AGE-BSA for the various time periods and analyzed by RT-PCR as described previously (22). 6-μl aliquots of each RT-PCR reaction mixture were electrophoresed on a 2% agarose gel and transferred to a Hybond-N+ nylon membrane, and the membrane was hybridized with the respective [32P]-end-labeled probes (20). 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 (20). Signal intensities of hybridized bands were measured by a

FIG. 1. SDS-PAGE of AGE-BSA. 10 μg of nonglycated native BSA (lane 1) and AGE-BSA (lane 2) were loaded on a 10% polyacrylamide gel with a stacking gel of 5% polyacrylamide. Staining of the gel was performed with Coomassie Brilliant Blue. Size markers (kDa) are shown on the left.

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RESULTS Characterization of AGE-BSA—AGE-BSA was prepared by incubating BSA with glucose and then purified by heparin-Sepharose CL-4B column chromatography. Fig. 1 shows its electrophoretic profile on reducing SDS-polyacrylamide gel electrophoresis. Control nonglycated BSA migrated to the position at 68 kDa. On the other hand, the purified materials migrated much more slowly, yielding a broad band larger than 68 kDa. This indicated that covalently linked adducts were formed nonenzymatically on BSA without discernible degrada-

2 The monoclonal antibody did not cross-react with basic fibroblast growth factor or platelet-derived growth factor. Its binding to VEGF-B (49) and -C (50), recently described members of the VEGF family, was undetectable and about 1/1000 of that to VEGF, respectively. VEGF-B and -C proteins were donated from the Ludwig Institute for Cancer Research and University of Helsinki.
tion. As shown in Table I, the purified materials also exhibited spectrophotometric features characteristic to AGE (26, 27). The peak fluorescence was noted at 440 nm with excitation at 370 nm, and its intensity was increased 10-fold in comparison with nonglycated BSA. Chromogen products also appeared in the purified materials, whereas they were barely detectable in nonglycated BSA. Based on these observations, the purified materials were used as AGE-BSA.

Characterization of Anti-AGE Antiserum—As a tool to evaluate AGE and their biological effects, an antiserum was raised against AGE-RNase A. The reactivity of this antiserum to several AGE-modified proteins, Amadori compounds, and carbamoyllysine derivatives was examined. Since it was possible that AGE-modified proteins might contain early glycation products, the immunoreactivity of the antiserum to reduced AGE-Hb was also tested because the early glycation products are known to be converted to glucitol-lysine by reduction with NaBH₄ (14, 28). As shown in Fig. 2A, there was no difference in the immunoreactivity in the noncompetitive ELISA between AGE-Hb and reduced AGE-Hb. Further, as shown in Fig. 2B, the antiserum binding to AGE-Hb was not competed for by 1-deoxy-1-propylamino-D-fructose in the competitive ELISA, indicating that the antiserum does not recognize Amadori compounds. We next tested whether the antiserum reacted to CM-TsLME and CM-BSA in the competitive ELISA. As shown in Fig. 2B and C, these glycoxidative products were found to partially inhibit the antiserum binding to reduced AGE-Hb, whereas AGE-Hb and AGE-RNase A fully inhibited its binding (Fig. 2D). Further, the antiserum reactivity to AGE-BSA was dependent on the duration of incubation of BSA with glucose (Fig. 2E). These results suggested that the antiserum could recognize AGE structures common to various AGE preparations.

**TABLE I**

<table>
<thead>
<tr>
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<th>Fluorescence</th>
<th>Chromogen products</th>
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<tr>
<td></td>
<td>arbitrary units</td>
<td>arbitrary units</td>
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<tr>
<td>BSA</td>
<td>1.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
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<tr>
<td>AGE-BSA</td>
<td>9.9 ± 0.1</td>
<td>0.11 ± 0.0</td>
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*a* An arbitrary value of 1 was assigned to fluorescence of control BSA. Fluorescence was measured at excitation of 370 nm and emission of 440 nm.

*b* Measured as absorbance at 350 nm/absorbance at 280 nm.

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**Fig. 2. Characterization of anti-AGE antiserum.** A, antiserum was titered in noncompetitive ELISA using Hb (○), AGE-Hb (●), and reduced AGE-Hb (▲) as absorvent antigens, as described under "Experimental Procedures." The amounts of the antigens are indicated on the abscissa, and absorbance at 450 nm is on the ordinate. B-D, antiserum was titered in competitive ELISA. Wells were coated with reduced AGE-Hb and then various test samples as competitor, and anti-AGE antiserum were added. B, 1-deoxy-1-propylamino-D-fructose (●); CM-TsLME (○). C, CM-BSA (●); 8-week incubated AGE-BSA (○). D, Hb (○); 8-week incubated AGE-Hb (●); 8-week incubated AGE-RNase A (▲). E, BSA (○); 4-week incubated AGE-BSA (●); 8-week incubated AGE-BSA (▲); 12-week incubated AGE-BSA (■). The amounts of the competitors are indicated on the abscissa, and absorbance at 450 nm is on the ordinate. Similar results were obtained in two independent experiments.
Poly(A) Forms of VEGF in Response to AGE—Endothelial cells obtained from human skin microvessels were cultured in the presence or absence of AGE-BSA, and the viable cell number was determined at days 1, 2, and 3 after the AGE addition. As shown in Fig. 3, AGE-BSA was found to increase the viable microvascular endothelial cell number in a dose-dependent manner; at 50 μg/ml AGE, there was about a 40% increase in viable cell number. Moreover, AGE-BSA significantly increased DNA synthesis in microvascular endothelial cells to 130% (p < 0.01, Fig. 4). However, nonglycated BSA induced no change in either the cell number or DNA synthesis.

Neutralization of the AGE-induced DNA Synthesis by Anti-AGE Antibody—To evaluate the specificity of the AGE-BSA effect on endothelial cell growth, we examined the effects of the antiserum against AGE-RNase A on AGE-induced DNA synthesis. As shown in Fig. 4, the anti-AGE-RNase A antiserum was found to completely neutralize the AGE-induced synthesis of endothelial cell DNA at 1%, while the same concentration of rat anti-rabbit IgG had no effect.

Microvascular Endothelial Cells Express mRNAs for Secretory Forms of VEGF in Response to AGE—Poly(A) mRNAs were isolated from microvascular endothelial cells treated with various concentrations of AGE-BSA for various time periods, and analyzed by a quantitative RT-PCR technique to determine the effects of AGE on the expression of the VEGF gene. It has been reported that there are four alternatively spliced products from the single VEGF gene, VEGF121, VEGF165, VEGF189, and VEGF206. Since Northern blot analysis cannot clearly discriminate the four mRNA species, we employed a more sensitive RT-PCR technique as described previously (20). In this experiment, 486- and 618-base pairs (bp)-long cDNA products would be amplified from mRNAs for VEGF121 and VEGF165, respectively (20).

As shown in Fig. 5, A and B, microvascular endothelial cells were expressing mRNAs for VEGF121 and VEGF165, the secretory forms of VEGF. When the endothelial cells were exposed to AGE-BSA, the level of VEGF mRNAs was found to be significantly increased in a time- and dose-dependent manner. The VEGF mRNA level began to increase at 2 h, and reached a maximum at 4 h in the presence of 50 μg/ml AGE-BSA; the peak value was 3-fold higher than the basal level when standardized with the signal intensities of β-actin mRNA as an internal control. Maximal stimulation was achieved at 50–100 μg/ml AGE. However, the larger alternatively spliced products coding for VEGF189 and VEGF206 were not detected in microvascular endothelial cells regardless of the presence or absence of AGE-BSA.

To confirm whether AGE-BSA increased the synthesis of VEGF proteins, we performed immunoprecipitation using the anti-VEGF monoclonal antibody (20) from lysates of the cells that had been treated with or without AGE-BSA. As shown in Fig. 5C, 35S-labeled proteins that migrated to the positions of 18 and 22 kDa, corresponding to VEGF121 and VEGF165, respectively, were immunoprecipitated, and the amounts of these proteins were found to be increased to about 2.5-fold by the AGE treatment.

VEGF Receptor Expressions in Microvascular Endothelial Cells—VEGF exerts its biological actions through its specific receptors, KDR and Flt 1 (31, 32). We then determined the types of VEGF receptors expressed in microvascular endothelial cells and whether their expressions could be altered by AGE-BSA. As shown in Fig. 5B, both kdr and flt 1 mRNA were detected in microvascular endothelial cells, and the content of kdr mRNA was more abundant than that of flt 1 mRNA. In contrast to VEGF mRNAs, the levels of the two types of receptors were essentially unchanged by the exposure to AGE-BSA.

Neutralization of the AGE-Induced DNA Synthesis of Microvascular Endothelial Cells by MoAb against Human VEGF—We next investigated whether vascular VEGF may have a functional role in the AGE action on endothelial cells. Microvascular endothelial cells were preincubated with various
concentrations of MoAb BL-2 for 30 min, then exposed to 50 μg/ml AGE-BSA for 24 h in the presence of the antibody, and assayed for [3H]thymidine incorporation. As shown in Fig. 6, MoAb BL-2 was found to significantly diminish the AGE-induced increase in DNA synthesis in a dose-dependent manner; at 10 μg/ml, a complete reversal was obtained. The MoAb alone did not affect DNA synthesis in endothelial cells not exposed to AGE.

**AGE Induction of Tube Formation of Microvascular Endothelial Cells and Its Inhibition by Anti-VEGF MoAb**—The process of angiogenesis has been assumed to be completed by the formation of microvascular tubes (19). In vitro assays for tube formation of endothelial cells have been developed and used to study this crucial step of angiogenesis. Accordingly, we examined whether AGE affect in vitro tube formation of microvascular endothelial cells. For this, we employed an on-gel assay system using Matrigel, in which endothelial cells take only several hours to associate with each together and form microtubes. Microvascular endothelial cells were seeded on Matrigel with or without AGE-BSA, and tube formations were judged after 6 h. As shown in Fig. 7A, AGE-BSA was found to double the length of the tubes of endothelial cells formed on Matrigel; and, the AGE-induced tube formation was inhibited by BL-2 MoAb as was the AGE-induced DNA synthesis of endothelial cells. The MoAb per se did not affect tube formation. Fig. 7B shows typical micrographs; with 10 μg/ml BL-2 MoAb, AGE-induced tube formation was markedly inhibited.

**DISCUSSION**

In the present study, we have demonstrated for the first time that AGE, nonenzymatically glycated protein derivatives formed under hyperglycemia, stimulate the growth and tube formation of human microvascular endothelial cells, the key steps of angiogenesis which take place in this very cell type (33, 34). The present findings have extended our preliminary work employing endothelial cells from a larger vessel, i.e. the umbilical vein. Though modestly, AGE-BSA caused a consistent increase in cell number and in DNA synthesis of both umbilical and microvascular endothelial cells, the same AGE-BSA concentration (50 μg/ml) giving the maximal effect in both cases (35).
This concentration of AGE was comparable with that of the *in vivO* situation in diabetes; Makita *et al.* reported (13) that human serum AGE levels were elevated more than 2-fold in diabetic patients (about 25 μg/ml) and almost 8-fold in diabetic patients on hemodialysis (about 80 μg/ml) in comparison with that in normal patients.

That it was AGE moieties that elicited the angiogenic activity was evidenced as follows. First, the AGE-BSA employed exhibited the biochemical hallmarks of AGE (Fig. 1 and Table I). Second, control nonglycated BSA made no change (data not shown). Third, a newly developed antiserum against AGE-RNase A could neutralize the growth-promoting effect of AGE-BSA (Fig. 4). Although this antiserum was partially reactive to carboxymethyllysine, it would seem unlikely that the AGE effect could be accounted for by such glycoxidative biproducts of the Maillard reaction, which might be present in trace amounts in the AGE-BSA preparation, because authentic carboxymethylated BSA added to the culture medium at concentrations from 1 to 100 μg/ml failed to stimulate the endothelial cell synthesis of DNA (data not shown). We speculate that the AGE actions on microvascular endothelial cells may require their binding to RAGE, the AGE-specific receptor, as is the case with bovine retinal pericytes (9), human umbilical vein endothelial cells (35), and human pancreatic cancer cells (36).

The present study has also demonstrated that the angiogenic activity of AGE is mainly mediated by autocrine VEGF synthesized by microvascular endothelial cells *per se*. mRNAs for VEGF121 and VEGF165 are present in microvascular endothelial cells, and their levels are up-regulated by AGE in both dose- and time-dependent manners (Fig. 5A and B). AGE did increase *de novo* synthesis of VEGF in endothelial cells (Fig. 5C). mRNAs for the two VEGF receptors, kdr and flt1, were also detected in human skin microvascular endothelial cells, their relative abundance being kdr >> flt1. However, their levels were essentially unaltered when exposed to AGE (Fig. 5B). This suggests that the ligand expression should be the rate-limiting step in the putative autocrine action of VEGF. In effect, the neutralization experiments established the functional role of VEGF in the AGE-induced endothelial cell growth and tube formation. MoAb against human VEGF could completely inhibit the AGE-induced tube formation as well as the DNA synthesis of microvascular endothelial cells (Figs. 6 and 7). Since the basal growth or tube formation in unexposed cells was not affected by the same concentration of the antibody, the antibody-induced inhibition is not likely the result of its toxic or nonspecific effects. These results thus indicate that autocrine VEGF is the main mediator of the AGE-driven angiogenesis *in vitro*.

In light of the present findings, together with the previous observations, we can now posit an overall scheme concerning the roles of AGE in the development of diabetic microangiopathy (Fig. 8). First, AGE act on pericytes, the microvascular constituent that encircles the endothelium. Through interactions with RAGE, AGE decrease the number of this cell type (9), leading to pericyte dropout, which would in turn relieve the restriction on endothelial cell replication and facilitate angiogenesis. The resultant cessation of pericyte-endothelial cell interactions would impair prostacyclin production (6), which would cause thrombogenesis. Second, AGE act on endothelial cells, which serve as a barrier between circulating blood and parenchyma and produced various vasoactive substances. As shown in this paper, one consequence is angiogenesis that is probably mediated by autocrine VEGF. In addition, the prostacyclin-synthesizing ability of microvascular endothelial cells might be directly inhibited by AGE, as in umbilical endothelial cells (35). Again, the consequence of this would be thrombogenesis. Diminished circulation or microthrombus formation may occur in such lesions, giving rise to hypoxia, the major factor triggering VEGF expression in both endothelial cells and pericytes (20, 37–40). In such circumstances, angiogenesis would further proceed, which may eventually lead to the clinical expression of diabetic microangiopathies, exemplified by proliferative retinopathy. According to this model, procedures that can halt those events, e.g., inhibition of AGE formation,^3^ AGE absorption by immobilized antibodies, antisense RAGE DNA (35, 36), prostacyclin analogues, or anti-VEGF neutralizing antibodies would then theoretically help circumvent the development and progression of diabetic microangiopathies.

Although this view basically stands on the *in vitro* experiments, in support are several *in vivo* or clinical observations.

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^3^ Sato *et al.* (51) have recently developed a novel class of inhibitors of AGE formation, which are distinct from aminoguanidine in both structure and mode of action.
Hammes et al. (41, 42) reported that aminoguanidine, an inhibitor of AGE formation, prevented AGE accumulation at branching sites of precapillary arterioles and that this inhibitor could diminish pericyte dropout and inhibit abnormal endothelial cell proliferation in streptozotocin-induced diabetic rats. Dolhofer-Bliesener et al. (43) have shown that, in human diabetic subjects, the serum level of AGE was associated with the state of late complications, particularly in cases with retinopathy. Wautier et al. (44) reported that infusion of diabetic red blood cells into normal rats can induce vascular hyperpermeability, which was completely inhibited by anti-RAGE IgG; they also demonstrated that an antioxidant, probucol, can similarly reverse the red blood cell transfer-induced vascular permeability, suggesting a role of AGE-RAGE interactions and the involvement of an oxidant stress-sensitive pathway in the development of hyperpermeability. Recent clinical studies at different institutions have established that intraocular concentrations of VEGF correlated with active neovascularization (45, 46). Further, the increased permeability of retinal capillaries and the breakdown of the blood-retinal barrier have been shown to be an early event in both human diabetic subjects and streptozotocin diabetic rats followed by new vessel formation (47, 48). The barrier breakdown would help ensure pericyte access to AGE. Moreover, it is reported that the barrier breakdown could be protected by the treatment of aminoguanidine, suggesting that AGE themselves may be directly involved in the barrier breakdown (48).

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