The binding of advanced glycation end products (AGE) to the receptor for AGE (RAGE) is known to deteriorate various cell functions and is implicated in the pathogenesis of diabetic vascular complications. Here we show that AGE, tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and 17\(\beta\)-estradiol (E\(_2\)) up-regulated RAGE mRNA and protein levels in human microvascular endothelial cells and ECV304 cells, with the mRNA stability being essentially invariant. Transient transfection experiments with human RAGE promoter-luciferase chimeras revealed that the region from nucleotide number −751 to −629 and the region from −239 to −89 in the RAGE 5′-flanking sequence, respectively, exhibited the AGE/TNF-\(\alpha\) and E\(_2\) responsiveness, respectively. Site-directed mutagenesis of an nuclear factor-\(\kappa\)B (NF-\(\kappa\)B) site at −671 or of Sp-1 sites at −189 and −172 residing in those regions resulted in an abrogation of the AGE/TNF-\(\alpha\) or E\(_2\)-mediated transcriptional activation. Electrophoretic mobility shift assays revealed that ECV304 cell nuclear extracts contained factors which retarded the NF-\(\kappa\)B and Sp-1 elements, and that the DNAs-protein complexes were supershifted by antibodies, respectively. These results suggest that AGE, TNF-\(\alpha\), and E\(_2\) can activate the RAGE gene through NF-\(\kappa\)B and Sp-1, enhancing AGE-RAGE interaction, which would lead to an exacerbation of diabetic microvasculopathy.

Glucose and other reducing sugars can react nonenzymatically with the amino groups of proteins to form reversible Schiff bases and, then, Amadori compounds. These early glycation products undergo further complex reactions to become irreversibly cross-linked, heterogeneous fluorescent derivatives termed advanced glycation end products (AGE)\(^1\) (1). AGE are known to accumulate in various tissues at an extremely accelerated rate under a diabetic state, and are implicated in the development of diabetic vascular complications, e.g. retinopathy and nephropathy (1). We have shown previously that AGE exert their effects on endothelial cells and pericytes, the constituents of microvessels, through interactions with a cell-surface receptor for AGE (RAGE); AGE stimulate the growth of microvascular endothelial cells through an induction of vascular endothelial growth factor (VEGF) leading to angiogenesis on the one hand (2), and inhibit prostacyclin production and stimulated plasminogen activator inhibitor-1 synthesis by the endothelial cells on the other (3). AGE exhibit a growth inhibitory action on pericytes (4), which would lead to pericyte loss, the earliest histological hallmark in diabetic retinopathy (5).

RAGE belongs to the immunoglobulin superfamily of cell surface molecules (6, 7). It is expressed in multiple tissues (8) and interacts with various ligands including AGE (9, 10). The engagement of RAGE by AGE has been reported to induce cellular oxidant stress, activating the transcription factor nuclear factor-\(\kappa\)B (NF-\(\kappa\)B) (11, 12), resulting in the perturbation of a variety of vascular homeostatic functions (9, 10). AGE-RAGE interaction thus has been thought to play a central role in the development of diabetic vasculopathy. To determine how the RAGE gene is regulated under a diabetic state is, therefore, important for clarifying the pathogenesis of diabetic complications as well as for understanding the physiological roles of RAGE.

It has been reported that AGE-rich blood vessels show enhanced RAGE immunoreactivity (13); this implies the possibility that AGE themselves may up-regulate the RAGE expression. Among cytokines, tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) is thought to be involved in the development of diabetes (14). Evidence has accumulated that serum TNF-\(\alpha\) levels are increased in non-insulin-dependent diabetes mellitus (15–17) and that TNF-\(\alpha\) can activate the NF-\(\kappa\)B pathway (18–20). Recently, NF-\(\kappa\)B has been reported to play a role in the basal and lipopolysaccharide-induced expression of the RAGE gene (21). It has also been reported that diabetic vasculopathy is often aggravated during pregnancy, probably due to the increased level of serum estrogen (22–24). From these observations, the possibility that TNF-\(\alpha\) and estrogen worsen the diabetic complications through the induction of RAGE gene expression should also be considered.

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\(^*\) This work was supported by Grant 97L00805 from the Research for the Future Program of the Japan Society for the Promotion of Science. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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\(^1\) The abbreviations used are: AGE, advanced glycation end products; RAGE, receptor for AGE; VEGF, vascular endothelial growth factor; NF-\(\kappa\)B, nuclear factor-\(\kappa\)B; TNF-\(\alpha\), tumor necrosis factor-\(\alpha\); E\(_2\), 17\(\beta\)-estradiol; BSA, bovine serum albumin; TGF-\(\beta\), transforming growth factor-\(\beta\)1; IFN-\(\gamma\), interferon-\(\gamma\); HMVEC, human skin microvascular endothelial cells; FBS, fetal bovine serum; PBS, phosphate-buffered saline; RT, reverse transcription; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; ER, estrogen receptor; ANOVA, analysis of variance.
Mechanisms of RAGE Gene Induction in Endothelial Cells

The present study, we thus examined the effects of AGE, TNF-α, and 17β-estradiol (E₂) on RAGE gene expression and found that the three agents are capable of up-regulating the RAGE mRNA and protein levels in human microvascular endothelial cells. On the other hand, non-glycated BSA, other cytokines, and an anti-estrogen did not affect the RAGE mRNA levels. mRNA stability and promoter assays demonstrated that the induction was at the transcriptional level, and that AGE and TNF-α induced the RAGE gene through an activation of NF-κB while E₂ induced the gene through Sp-1.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine serum albumin (BSA) (fraction V, fatty acid-free, endotoxin-free) was purchased from Roche Molecular Biochemicals (Mannheim, Germany). TNF-α was purchased from Becton Dickinson Labware (Bedford, MA). Transforming growth factor-β1 (TGF-β1) and interferon-γ (IFN-γ) were from R&D Systems, Inc. (Minneapolis, MN). E₂ and 4-hydroxytamoxifen (4-OH tamoxifen) were from Sigma. Ascorbic acid, lipopolysaccharide (LPS), heparin (10 mg/ml), amphotericin B (50 ng/ml), basic fibroblast growth factor (5 ng/ml), and hydrocortisone, and FBS, respectively. These media were dialyzed against phosphate-buffered saline (PBS), glucose-modified BSA with 0.5 M glucose at 37 °C for 6 weeks under sterile conditions as described previously (2).

**Preparation of AGE-BSA**—AGE-BSA was prepared by incubating BSA with 0.5 M glucose at 37 °C for 6 weeks under sterile conditions, using a Quick Prep Micro mRNA purification kit (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom), and analyzed by RT-PCR with a GeneAmp RNA PCR kit (Perkin-Elmer, Foster City, CA) as described previously (27). Oligodeoxyribonucleotide primers and probes for human RAGE, VEGF, and β-actin mRNA were the same as described previously (4, 28). The amounts of poly(A)⁺ RNA resultant band with cycle number, for determination were chosen in quantitative range of reactions proceeded linearly as described previously (28, 29); 30 ng of templates and 30 cycles were chosen for amplifying human RAGE mRNA, 30 ng and 40 cycles for human VEGF mRNA, and 30 ng and 20 cycles for β-actin mRNA. The fragments amplified with PCR were sequence-verified on both strands by the chain termination method (30). Five-μl aliquots of each RT-PCR reaction mixture were electrophoresed on a 2% agarose gel and transferred to a Hybond-N⁺ nylon membrane (Amersham Pharmacia Biotech), and the membrane was hybridized with the respective probes, which had been 32P-end-labeled with polynucleotide kinase (27). Signal intensities of hybridized bands were measured by a BAS 1000 BioImage analyzer (Fuji Photo Film Co., Ltd., Hamamatsu, Japan).

**Analysis of RAGE mRNA Stability**—HMVEC or ECV304 cells were treated with TNF-α, E₂, or AGE-BSA for 4 h, and further cultured in the absence of glucose. The concentration of AGE-BSA and control BSA were determined by the method of Bradford (26). The concentration of AGE-BSA and control BSA were determined by the method of Bradford (26).

**Measurement of RAGE mRNA by Quantitative Reverse Transcription-PCR (RT-PCR)**—Poly(A)⁺ RNAs were isolated from subconfluent cultures of HMVEC or ECV304 cells incubated under various conditions, using a Quick Prep Micro mRNA purification kit (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom), and analyzed by RT-PCR with a GeneAmp RNA PCR kit (Perkin-Elmer, Foster City, CA) as described previously (27). Oligodeoxyribonucleotide primers and probes for human RAGE, VEGF, and β-actin mRNA were the same as described previously (4, 28). The amounts of poly(A)⁺ RNA resultant band with cycle number, for determination were chosen in quantitative range of reactions proceeded linearly as described previously (28, 29); 30 ng of templates and 30 cycles were chosen for amplifying human RAGE mRNA, 30 ng and 40 cycles for human VEGF mRNA, and 30 ng and 20 cycles for β-actin mRNA. The fragments amplified with PCR were sequence-verified on both strands by the chain termination method (30). Five-μl aliquots of each RT-PCR reaction mixture were electrophoresed on a 2% agarose gel and transferred to a Hybond-N⁺ nylon membrane (Amersham Pharmacia Biotech), and the membrane was hybridized with the respective probes, which had been 32P-end-labeled with polynucleotide kinase (27). Signal intensities of hybridized bands were measured by a BAS 1000 BioImage analyzer (Fuji Photo Film Co., Ltd., Hamamatsu, Japan).

**Analysis of RAGE mRNA Stability**—HMVEC or ECV304 cells were treated with TNF-α, E₂, or AGE-BSA for 4 h, and further cultured in the absence of 10 μM of actinomycin D (Sigma) for various time periods. Total RNAs were isolated from the cultures with Isogen (Nippon Gene, Toyama, Japan) according to the method described by Chomczynski and Sacchi (31), and analyzed by the quantitative RT-PCR method described above. The amounts of total RNA templates and cycle numbers for amplification were chosen in quantitative ranges; 300 ng of templates and 35 cycles and 30 ng of templates and 40 cycles for amplifying human RAGE mRNA, and 300 ng and 20 cycles for β-actin mRNA.

**Western Blot Analysis**—Subconfluent cultures of HMVEC or ECV304 cells were incubated with TNF-α, E₂, or AGE-BSA for 24 h. After the incubation, cells were washed with cold PBS, scraped off in cold PBS, and pelleted by centrifugation at 300 × g at 4 °C for 5 min. The cells were lysed immediately by sonication in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.002% bromphenol blue) and boiled at 95 °C for 5 min. Twelve μg of the cell lysates were resolved by SDS-PAGE (12.5%), and then transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech). The membrane was treated with the anti-RAGE polyclonal antibodies, and the immunoreactive bands were visualized with an ECL detection system (Amersham Pharmacia Biotech) as described previously (32). Signal intensities of hybridized bands were determined densitometry using BIO-PROFIL 1-D (version 5.08) software (Vilber Lourmat Biotechnology, Marne La Vallée, France).

**Construction of the RAGE Promoter-Luciferase Chimeras**—The chimeric genes for transfection experiments were constructed by ligating the 5'-flanking regions of differing lengths of the human RAGE gene upstream of the luciferase gene in a pGL3-basic vector (Promega Corp., Madison, WI) (see Fig. 5A). The genomic DNA fragment of the human RAGE gene were amplified by PCR using a cosmid named K571 (33) as a template, which was kindly provided by Professor Toshimichi Ike-mura (National Institute of Genetics, Shizuoka, Japan). The PCR primers employed in the amplification reactions are shown in Table 1. Furthermore, we constructed two additional chimeric genes. The DNA fragments containing exons 1-11 plus introns 1-10 or the 3'-flanking region of the human RAGE gene were amplified by PCR using the

**TABLE I**

Primers used for the construction of RAGE promoter-luciferase fusion genes

<table>
<thead>
<tr>
<th>Forward primers</th>
<th>Nucleotide sequences</th>
<th>Corresponding nucleotide positions in the RAGE gene (33)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGL-1</td>
<td>5'-GACGCGGT AGAGATTGCCAAAATGGGGA</td>
<td>-1689 to -1669</td>
</tr>
<tr>
<td>pGL-2</td>
<td>5'-GACGCGGT CCTTACTTATACGGCACGCTCA</td>
<td>-1599 to -1579</td>
</tr>
<tr>
<td>pGL-3</td>
<td>5'-GACGCGGT TACCTGGGAGGAAAGTTCTG</td>
<td>-1528 to -1508</td>
</tr>
<tr>
<td>pGL-4</td>
<td>5'-GACGCGGT GGGATATGGAGGTGGGCTC</td>
<td>-1490 to -1480</td>
</tr>
<tr>
<td>pGL-5</td>
<td>5'-GACGCGGT ACTGGCCCATCCTACGCCCT</td>
<td>-751 to -731</td>
</tr>
<tr>
<td>pGL-6</td>
<td>5'-GACGCGGT TTTGAACTGTTATGGTGGGG</td>
<td>-629 to -609</td>
</tr>
<tr>
<td>pGL-7</td>
<td>5'-GACGCGGT GAGACCTTCAAGGTTGACACT</td>
<td>-239 to -219</td>
</tr>
<tr>
<td>pGL-8</td>
<td>5'-GACGCGGT CGACTGAAAGATGGGCGCT</td>
<td>-89 to -69</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>5'-GAGAATCT CAGGCTCCAACTGGTTGCC</td>
<td>+43 to +23</td>
</tr>
<tr>
<td>pGL-1–8</td>
<td>5'-GAGAATCT CAGGCTCCAACTGGTTGCC</td>
<td>+43 to +23</td>
</tr>
<tr>
<td>Forward primers</td>
<td>5'-GAGTCGAC TCTTCAAGCTGTTGTTGAGG</td>
<td>+52 to +72</td>
</tr>
<tr>
<td>pGL-3fl</td>
<td>5'-GAGTCGAC CCCCCAAATGTAGTAAACC</td>
<td>+3253 to +3273</td>
</tr>
<tr>
<td>Reverse primers</td>
<td>5'-GAGTCGAC AGACGAGATGGTGCAGTG</td>
<td>+3272 to +3252</td>
</tr>
<tr>
<td>pGL-2fl</td>
<td>5'-GAGTCGAC AGACGAGATGGTGCAGTG</td>
<td>+3272 to +3252</td>
</tr>
<tr>
<td>pGL-3fl</td>
<td>5'-GAGTCGAC AAGAGTGGTACAAGAGAAG</td>
<td>+6318 to +6298</td>
</tr>
</tbody>
</table>
cosmid KS71 and specific primers shown in Table I, and were ligated to downstream of the luciferase gene in the pGL3-Basic vector that had carried the longest fragment of the 5'-flanking region (see Fig. 5A). All the fragments obtained were sequence-verified.

Site-directed Mutagenesis—Mutations to be introduced into the RAGE promoter-coding region were generated using a GeneEditor™ in vitro site-directed mutagenesis system (Promega) according to the manufacturer’s protocol. The nucleotide sequences of the mutagenic oligodeoxyribonucleotide primers were 5′-ACTGTCAGAGTTGGGTCTCCCTC-TCCATTAAAGGC (nucleotides −683 to −662), 5′-CTGTCAGGTTCTACCAAGAACCTCTTCCCTGAG-3′ (−202 to −171), 5′-CTGTCAGGTTCTACCAAGAACCTCTTCCCTGAGA-3′ (−154), 5′-CTGTCAGGTTCTACCAAGAACCTCTTCCCTGAGAA-3′ (−199 to −179) for yielding pGL-5 NF-B2m, pGL-7 Im, pGL-7 IIm, or pGL-7 IIm, respectively (mutated sites are indicated by underlines) (see schematic representations in Fig. 6A). pGL-5 NF-B2m contained the region −751 to −43, but with the mutation in an NF-B site (−671 to −663), pGL-7 IIm and pGL-7 IIm contained the region −239 to +43, but with the mutations in one of two Sp-1 sites (−189 to −181 and −172 to −166), respectively. pGL-7 IIm IIm had the mutations in both Sp-1 sites. All the mutated constructs were sequence-verified.

Transfection Experiments and Luciferase Assay—ECV304 cells or HMVEC (2 × 10^5 cells) were plated into the wells of six-well tissue culture plates (Becton Dickinson Labware) 1 day before transfection. For transfection the DNA/cationic lipid mixture for transfection was then added to a tube containing 100 μl of Opti-MEM I (Life Technologies, Inc.), TransFast™ transfection reagent (7.5 μl) (Promega), and 1 ml of the experimental medium. Cells were exposed to the DNA/cationic lipid mixture for 2 h, then received 5 ml of the FBS-containing medium and were further incubated for 36 h at 37 °C. For transfection of HMVEC, test plasmid (2 μg) and pRL-CMV vector (1 μg) (Promega) were added to a tube containing 100 μl of Opti-MEM I (Life Technologies, Inc.). Lipofectin reagent (6 μl) (Life Technologies, Inc.) was added to another tube containing 100 μl of Opti-MEM I. The plasmid DNA and lipofectin reagent were then mixed together. After incubation at room temperature for 30 min, DNA-lysosome complex was diluted with 800 μl of Opti-MEM I. Cells were exposed to the mixture for 8 h, then received 2 ml of the FBS-containing medium and were further incubated for 36 h at 37 °C. After the incubation, the cells were treated with TNF-α, E2, or AGE-BSA for 8 h in the experimental medium. Luciferase activities were measured using a Dual-Luciferase® reporter assay system (Promega) according to the manufacturer’s protocol with a luminometer (Fluoroskan Ascent FL version 2.2.4, Labsystems, Helsinki, Finland).

Preparation of Nuclear Extracts from Cultured Cells—Nuclear extracts were prepared essentially as described by Schreiber et al. (34). Briefly, ECV304 cells (2 × 10^6) were plated onto 75-cm² tissue culture flasks (Becton Dickinson Labaware) in the complete medium and left for 24 h at 37 °C. The cells were further incubated in the experimental medium at 37 °C for 12 h and then treated with TNF-α, E2, or AGE-BSA for 4 h. After the treatment, the cells were washed twice with ice-cold PBS (10 ml), and homogenized by centrifugation at 15,000 rpm in a Beckman GH3.7 rotor at 4 °C for 5 min. The pellet cells were resuspended in 0.4 ml of ice-cold buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) and placed on ice for 15 min. After the addition of 25 μl of 10% Nonidet P-40, the suspension was vortexed for 15 s and centrifuged at 13,000 rpm in a Hitachi T155 rotor at 4 °C for 30 min. The resultant nuclear pellets were washed with buffer A and resuspended in 0.1 ml of a solution containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride by constant agitation for 15 min at 4 °C. The lysates were centrifuged at 13,000 rpm at 4 °C for 10 min, and the supernatants were collected as nuclear extracts. The protein concentrations of the nuclear extracts were determined by the method of Bradford (26).

Electrophoretic Mobility Shift Assay and Supershift Assay—The wild type and mutant double-stranded oligodeoxyribonucleotides encompassing the NF-κB site (nucleotide numbers −671 to −663) or the two Sp-1 sites (−189 to −181 and −172 to −168) were prepared. Their sequences were 5′-AGCTGGGGTCTCCCTGAGAATGGGGTGATAATTAT-3′ and 5′-AGCTGGGGTCTCCCTGAGAATGGGGTGATAATTAT-3′. For electrophoretic mobility shift assay (Fig. 2A), the probes were labeled with [γ-32P]ATP by T4 polynucleotide kinase (27) and incubated with the antibodies to NF-κB, E2, or AGE-BSA for 2 h in a binding buffer (0.25 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 0.5 mM phenylmethysulfonyl fluoride) by constant agitation for 15 min, and then incubated with the labeled wild-type probe for 20 min under the same conditions as described above. For supershift assays, antibodies to NF-κB p65, NF-κB p50, Sp-1, estrogen receptor (ER) α, or ERβ (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were added to the nuclear extracts and incubated at 4 °C for 12 h. Three independent nuclear extracts were subsequently incubated with the labeled oligodeoxyribonucleotides for 30 min under the same conditions as described above.

Statistical Analysis—Paired t tests and one-way analysis of variance (ANOVA) with Tukey’s range tests were used to test for significant differences between groups. All experiments were carried out at least three times.

RESULTS

TNF-α, E2, and AGE-BSA Increased the RAGE mRNA and Protein Levels in HMVEC and ECV304 Cells—To examine the effects of TNF-α, E2, and AGE-BSA on the RAGE mRNA level in HMVEC, poly(A)⁺ RNAs were isolated from cells that had been exposed to various concentrations of these agents for 4 h, and analyzed by the quantitative RT-PCR method. As shown in Fig. 1A, TNF-α, E2, and AGE-BSA increased the RAGE mRNA levels in dose-dependent manners. The extents of induction and the peak concentrations were about 3-fold at 100 ng/ml TNF-α, 10 μg/ml E2, and 50 μg/ml AGE-BSA, respectively, when normalized by β-actin mRNA-derived signals used as an internal control. Next, we examined the time course of the RAGE mRNA induction. HMVEC were treated for various time periods with TNF-α, E2, and AGE-BSA at their most effective doses. As shown in Fig. 1B, the mRNA levels began to increase at 2 h and reached a maximum at ~8 h after the addition of either of the three agents. On the other hand, exposure of HMVEC to TGF-β1 (10 ng/ml), IFN-γ (165 ng/ml), and non-glycated BSA (50 μg/ml) for 4 h did not affect the RAGE mRNA levels (Fig. 1C). 4-OH tamoxifen (10 μM), an anti-estrogen, abolished the E2-induced RAGE mRNA induction (Fig. 1C).

We next determined the RAGE mRNA levels in ECV304 cells, an immortalized cell line derived from human umbilical vein endothelial cells (25). This cell line exhibited the same responsiveness to AGE-BSA with respect to VEGF induction (Fig. 2A) as did HMVEC (2), the most effective dosage of AGE-BSA being 50 μg/ml in both cultures. The extent of induction was about 4-fold. When exposed to TNF-α (100 ng/ml), E2 (10 ng/ml), or AGE-BSA (50 μg/ml) for 4 h, the RAGE mRNA levels in the ECV304 cells were also increased about 2-fold compared with those in the control unexposed cells (Fig. 2B).

We next examined whether the increase in RAGE mRNA was actually followed by an increase in RAGE proteins in HMVEC and ECV304 cells. The cells were treated with TNF-α, E2, or AGE-BSA for 24 h, and subjected to Western blot analysis with anti-RAGE polyclonal antibodies. As shown in Fig. 3, one major immunoreactive band was marked at 46 kDa in either HMVEC (Fig. 3A) or ECV304 cells (Fig. 3B), being consistent with our previous report (3), and its intensity was increased by the treatment with the three agents. The RAGE protein levels in HMVEC and ECV304 cells treated with TNF-α, E2, or AGE-BSA were about 2-fold higher than the basal levels. The results indicated that ECV304 cells retained the ability to respond to those agents as did primary cultured endothelial cells, and subsequently we mainly used this cell line to examine the mechanisms of RAGE gene induction.

Effects of TNF-α, E2, and AGE-BSA on RAGE mRNA Stability in HMVEC and ECV304 Cells—We next determined the RAGE mRNA stability in HMVEC and ECV304 cells exposed to 5 μg of nuclear extracts at room temperature for 30 min. Samples were then loaded onto 6% polyacrylamide gels and run in 0.2× Trisborate/EDTA electrophoresis buffer at 10 V/cm for 2–3 h. The gels were dried and autoradiographed at ~80 °C overnight. For competition assays, nuclear extracts were first incubated with a 50-fold excess of unlabeled wild-type or mutant oligodeoxyribonucleotides at room temperature for 15 min, and then incubated with the labeled wild-type probe for 30 min under the same conditions as described above. For supershift assays, antibodies to NF-κB p65, NF-κB p50, Sp-1, estrogen receptor (ER) α, or ERβ (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were added to the nuclear extracts and incubated at 4 °C for 12 h. Three independent nuclear extracts were subsequently incubated with the labeled oligodeoxyribonucleotides for 30 min under the same conditions as described above.
or not exposed to the three agents to determine which step of gene expression accounted for the increase in RAGE mRNA levels. The cells were incubated in the presence or absence of TNF-α, E2, or AGE-BSA for 4 h, then incubated with actinomycin D for various time periods, and underwent quantitative RT-PCR analyses. As shown in Fig. 4 (A and B), the half-lives of RAGE mRNA in TNF-α-, E2-, and AGE-BSA-treated or untreated HMVEC and ECV304 cells were calculated from the RAGE and β-actin mRNA-derived signals to be between 2.1 and 2.8 h, and there was no statistically significant difference among them. The results suggested that the TNF-α-, E2-, and AGE-BSA-induced increase in RAGE mRNA was achieved at the transcriptional level.

Identification of the cis-Elements Responsive to TNF-α, E2, and AGE-BSA in the RAGE Promoter—To confirm whether TNF-α, E2, and AGE-BSA did induce the RAGE gene transcription and, if so, to delimit the regions involved in such transcriptional activations, a series of chimeric 5'-deletion promoter-luciferase reporter constructs were prepared. Schematic representations of the constructs are shown in Fig. 5A. ECV304 cells were transiently transfected with the constructs, and the effects of TNF-α (Fig. 5B), E2 (Fig. 5C), or AGE-BSA (Fig. 5D) on the luciferase activity in the transfected cells were determined. pGL-1 carried the longest 5'-flanking region of the RAGE gene (1689 base pairs upstream of the transcription start site; Ref. 14), and when the pGL-1-transfected cells were exposed to TNF-α (Fig. 5B), E2 (Fig. 5C), or AGE-BSA (Fig. 5D), the promoter activities (closed columns) increased significantly (1.5- to 2-fold) compared with those in unexposed cells (open columns). The same con-
concentration of non-glycated BSA did not induce the luciferase activity in the transfected cells (data not shown). Deletion of the 5′-flanking region of the RAGE gene to −751 (pGL-2 to -5) did not affect the TNF-α- and AGE-induced luciferase expression, but deletion to −629 (pGL-6) abolished the induction (Fig. 5, B and D). In contrast, the construct with a deletion to −239 (pGL-7) still retained the E2-induced luciferase expression but deletion to −89 (pGL-8) abolished the induction (Fig. 5C). The −751 to −629 region contained an NF-κB site (−671 to −663), and the −239 to −89 region contained two Sp-1 sites (−218 to −211 and −169 to −166). The results thus suggested that the NF-κB site and the Sp-1 sites might be required for the TNF-α/AGE- and E2-induced activation of the RAGE gene promoter, respectively.

We also tested additional constructs that carried genomic fragments containing exons 1–11 and introns 1–10 or the 3′-flanking region (pGL-e1–11 and pGL-3′fl, respectively) in addition to the pGL-1 5′-promoter region. As shown in Fig. 5E, when the pGL-e1–11- or pGL-3′fl-transfected cells were stimulated by TNF-α (closed columns), E2 (lattice columns), or AGE-BSA (dotted columns), the extents of luciferase induction were almost indistinguishable from those in the pGL-1-transfected cells. The results indicated that there were neither stimulatory nor silencing elements in the exon/intron or 3′-flanking region of the RAGE gene that could affect its responsiveness to TNF-α, E2, or AGE-BSA.

To determine the role of the NF-κB site at −671 to −663 in the TNF-α and AGE activation of the RAGE promoter, site-directed mutagenesis was performed at that site (pGL-5 NF-κB2m) (Fig. 6A). When luciferase activities were assayed in cells transfected with the mutant, the inducibility by both TNF-α and AGE-BSA was found to be totally abolished (Fig. 6B). Similarly, we also determined the role of the two Sp-1 sites at −189 to −181 and −172 to −166 in the E2-dependent transcriptional activation. We altered each of the two Sp-1 sites (pGL-7 Im and pGL-7 IIm) or both sites (pGL-7 ImIIm) (Fig. 6A), and the E2 effect on luciferase activities in the mutant transfectants was tested (Fig. 6C). The E2 inducibility of
Luciferase activities was decreased in pGL-7 Im- and pGL-7 IIm-transfected cells and totally abolished in pGL-7 IImIIm-transfected cells. The results indicated that the NF-κB binding site and the two Sp-1 binding sites were essential for the TNF-α/AGE- and E2-induced activation of the RAGE gene, respectively.

Fig. 5. Construction of RAGE promoter luciferase-chimeras and identification of TNF-α, E2, or AGE-BSA-responsive regions. A, schematic representation of the 5′-deleted RAGE promoter-luciferase reporter fusion gene constructs. Transcription start site (14) is designated as +1. Numbers in parentheses indicate nucleotide positions 5′ to the transcription start site. B–D, relative luciferase activities in ECV304 transfectants treated with TNF-α (100 ng/ml) (B), E2 (10 nM) (C), and AGE-BSA (50 μg/ml) (D). Open and closed columns indicate the mean values of untreated and treated cells, respectively. Data were normalized by pRL-SV40-derived luciferase activities used as an internal control, and related to the value of untreated pGL-1-transfected cells. Bars, standard deviations of nine independent experiments. E, relative luciferase activities in ECV304 cells transiently transfected with pGL-1, pGL-e1–11, or pGL-3 fl in the absence (open columns) or presence of TNF-α (100 ng/ml) (closed columns), E2 (10 nM) (lattice columns), or AGE-BSA (50 μg/ml) (dotted columns). Data were normalized by the activities derived from the internal control. Values are shown as the mean ± S.D. of four independent experiments. Statistical analysis was performed using ANOVA. B, pGL-1, -2, -3, -4, and -5 ± TNF-α, p < 0.01; pGL-6, -7, -8, and pGL3-basic ± TNF-α, not significant. C, pGL-1, -2, -3, -4, -5, -6, -7 ± E2, p < 0.01; pGL-8 and pGL3-basic ± E2, not significant. D, pGL-1, -2, -3, -4, -5 ± AGE-BSA, p < 0.01; pGL-6, -7, -8, and pGL3-basic ± AGE-BSA, not significant. E, there were no significant differences in relative luciferase activities among cells transfected with pGL-1, pGL-e1–11, and pGL-3 fl, which received or did not receive TNF-α, E2, or AGE-BSA, while p < 0.01 was noted between control versus each treatment within each construct.
and the Sp-1 sites (Fig. 7C, lane 3). In contrast, preincubation with mutant probes had little effect on the binding to the NF-κB and Sp-1 sites (Fig. 7, A–C, lane 4).

Next, we examined by supershift assays which members of the NF-κB family were responsible for the stimulation of the RAGE gene expression by TNF-α and AGE-BSA. When the nuclear extracts from the cells exposed to TNF-α (Fig. 7D) or AGE-BSA (Fig. 7E) were incubated with either anti-p65 or anti-p50 antibody, a more slowly migrating band (Fig. 7, B and E, arrow) newly appeared with a concomitant decrease in the original complex (Fig. 7, A and E, closed arrowhead). We also examined which factors were involved in the E2 induction of the RAGE gene. When the nuclear extracts from the cells treated with E2 (Fig. 7F) were incubated with either anti-Sp-1 or anti-ERα antibody, the original DNA-protein complex was supershifted upward (Fig. 7F, lanes 2 and 3), whereas anti-ERβ antibody gave no effect (Fig. 7F, lane 4). The results indicated that the DNA-binding complex for the NF-κB site was composed mainly of p50 and p65 members of the NF-κB family, and that the DNA-binding complex for the Sp-1 sites was composed mainly of Sp-1 and ERα.

The NF-κB and Sp-1 Sites Also Mediated the TNF-α/AGE-BSA- and E2-induced RAGE Gene Expression in HMVEC—To confirm whether the NF-κB site and Sp-1 sites are also involved in the TNF-α/AGE-BSA- and E2-induced up-regulation of RAGE gene in normal diploid microvascular endothelial
cells, we performed luciferase assays in HMVEC using pGL-5 and its mutants for the TNF-α/AGE-BSA-induced transcriptional activation, and pGL-7 and its mutants for the E2-induced activation. As shown in Fig. 8A, TNF-α and AGE-BSA induced luciferase expression 3-fold in pGL-5-transfected cells, the extent of induction being comparable with those of the RAGE mRNA induction in HMVEC (Fig. 1). The inducibility by both TNF-α and AGE-BSA was found to be totally abolished in pGL-5 NF-κBm- or pGL-6-transfected cells (Fig. 8A). E2 induced luciferase expression about 2.6-fold in pGL-7-transfected cells (Fig. 8B). This extent of induction was also comparable with that of the RAGE mRNA induction by E2 in HMVEC (Fig. 1). The E2 inducibility of luciferase activities was totally abolished in pGL-7 ImIm- or pGL-8-transfected cells (Fig. 8B).

**DISCUSSION**

We (2–4, 35) and others (36–39) have shown previously that interactions between AGE and RAGE cause phenotypic changes in microvascular endothelial cells and pericytes that are characteristic of diabetic vasculopathy. In this paper, we have demonstrated for the first time that AGE, the RAGE ligand itself, TNF-α, and E2 specifically up-regulate RAGE mRNA and protein levels in human vascular endothelial cells, and that this process is mediated by two distinct nuclear complexes, namely p65/p50 NF-κB and Sp-1/ERα.

It has been reported that AGE-rich vasculature exhibits enhanced RAGE immunoreactivity in the sites of diabetic microvascular injury (13). This finding suggested that AGE themselves could activate the RAGE gene expression, and that increased RAGE might further transduce AGE signals within microvascular cells. The present study has clearly shown that AGE-BSA did, but non-glycated BSA did not, up-regulate the RAGE gene expression and that this was achieved at the level of transcription, because RAGE mRNA half-lives were unchanged by the treatment with AGE (Fig. 4), and because AGE elicited the expression of the reporter gene linked to the 5′ promoter region of the RAGE gene (Fig. 5). The experiments with the RAGE promoter-reporter gene constructs demonstrated the presence of an AGE-responsive element in the −751 to −629 region of the human RAGE gene, and that an NF-κB site residing at −671 conferred the responsiveness to AGE on the RAGE gene (Figs. 5, 6, and 8). Further, the AGE-dependent formation of the NF-κB element-p65/p50 complex was demonstrated (Fig. 7). These results are considered to be consistent with the recent observations by others (11, 12) that AGE engagement of RAGE induces cellular oxidant stress, thereby activating the transcription factor NF-κB. The fact that AGE themselves induce the RAGE gene appears to be important when considering the mechanisms of development of diabetic vascular complications. Such a positive feedback loop in the diabetic state may exacerbate diabetic vasculopathy, exemplified by retinopathy and nephropathy.

We also demonstrated that TNF-α is another inducer of the RAGE gene (Figs. 1–3). TNF-α is known to be overexpressed in adipose tissue under obese and diabetic states (15–17) and to cause insulin resistance, the central and early component of non-insulin-dependent diabetes mellitus (18). TNF-α affects not only insulin sensitivity by suppressing insulin-stimulated tyrosine phosphorylation of insulin receptor substrate-1 (40) but also cell survival by NF-κB activation (20–22). We thus propose that an increased TNF-α level in non-insulin-dependent diabetes mellitus patients may worsen diabetic vasculopathy via RAGE gene induction. The TNF-α-induced stimulation of human RAGE gene was found to be also transcriptional and to be achieved by the same NF-κB element and binding complex (Figs. 5–8) as was the AGE stimulation. Recently, Li et al. (14) reported that the RAGE gene was activated by lipopolysaccharide via NF-κB sites at −671 and −467 in bovine aortic endothelial cells and rat vascular smooth muscle cells, and that both sites were required for full activation by lipopolysaccharide. Our results indicated that only one NF-κB site at −671 was required for RAGE gene induction by AGE-BSA and TNF-α in human microvascular endothelial cells (Figs. 5, 6, and 8). The discrepancy may be due to differences in the species or ligand specificity of the gene activation. The results suggest that the factors that have the ability to activate the NF-κB pathway can induce RAGE gene expression and have the potential to aggravate the AGE-mediated diabetic complications.

Pregnancy is known to worsen diabetic retinopathy (22–24). Schocket et al. (41) showed that a decrease in the retinal volumetric blood flow in diabetic patients during pregnancy might exacerbate retinal ischemia and aggravate retinopathy. Suzuma et al. (42) demonstrated that E2 at the concentration often observed during pregnancy (~10 nM) stimulates VEGF-dependent angiogenesis through the up-regulation of VEGF receptor-2 expression. However, the mechanisms underlying the adverse effects of E2 on diabetic complications are not yet fully understood. In this study, we demonstrated that E2 is an alternative inducer of the RAGE gene in human endothelial cells, enhancing its transcription at the concentration of 10 nM (Figs. 1–3). An anti-estrogen, 4-OH tamoxifen, totally abolished the E2-induced RAGE mRNA induction in HMVEC, while itself caused no change in the RAGE mRNA level (Fig.
This was regarded as an indication that E2 may act on RAGE gene through an estrogen receptor. The RAGE promoter does not contain any classical estrogen-responsive element (43) but contains several GC-rich boxes, which can bind to the transcription factor Sp-1 (14, 33, 44). Clearly, E2 utilizes a device that is different from those employed by AGE and TNF-α in the induction of RAGE gene. Two Sp-1 binding sites at −189 and −172 and an Sp-1/ER complex were involved in the E2 activation, and full E2 responsiveness required both cis-acting elements (Figs. 5–8). Recent studies from other laboratories have shown that an interaction of Sp-1/ER complex with GC-rich motifs in the promoter region is required for the transcriptional activation of several E2-responsive genes (45–49). The results obtained in this study suggest that the E2 induction of RAGE may partly underlie the exacerbation of diabetic retinopathy during pregnancy.

There are many genes that are regulated by the combination of NF-kB and Sp-1, and in some cases a direct interaction between NF-kB protein and Sp-1 protein has been demonstrated (50, 51). In the induction of the RAGE gene, however, such a direct interaction between the two factors would seem unlikely to occur because the Sp-1-mediated E2 responsiveness was still retained in the constructs with the deletion of the more than 500-nucleotide region encompassing the NF-κB element (Fig. 5). Cytokines that did not affect the endothelial cell expression of RAGE gene included TGF-β1 and IFN-γ (Fig. 1C). This indicated that Smad (52) or Janus kinase-signal transducers and activators of transcription (53) systems would not be involved in the regulation of RAGE gene.

In summary, we found that AGE and TNF-α enhanced RAGE expression through activation of the p65/p50 complex of NF-κB, and that E2 also activated RAGE expression through activation of the Sp-1/ER complex. Although the extent was rather modest, the three agents consistently increased the RAGE mRNA and protein levels in vascular endothelial cells. Chronic exposure to AGE, TNF-α, and/or E2 and sustained enhancement of RAGE expression may cause a further accumulation of AGE in the vasculature, resulting in an exacerbation of AGE-RAGE-mediated vascular dysfunctions. Such mechanisms of RAGE gene activation probably have evolved to regulate functions of this multifunctional receptor in various bio-logic processes, such as neurite formation of cortical neurons mediated by amphotericin engagement (54) and proinflammatory responses mediated by a recently discovered endogenous ligand, EN-RAGE (55). Obviously, diabetes abates the molecular devices for the RAGE gene regulation, resulting in the formation of a vicious circle which may eventually lead to the development and progression of diabetic vasculopathy. The enhanced interaction between AGE and RAGE may further increase VEGF expression in endothelial cells and/or retinal pigment epithelial cells (2, 56), resulting in angiogenesis, and also inhibit pericyte growth, leading to pericyte loss (4). Although more studies are needed to better clarify the significance of the RAGE gene activation by AGE, TNF-α, and E2 as well as immediate post-RAGE signaling events that lead to NF-κB and Sp-1 activations, inhibition of the RAGE gene activation may become a promising target for the prevention and treatment of diabetic vascular complications.

Acknowledgments—We thank Shin-ichi Matsudaira, Reiko Kitamura, and Tomoko Yachi for assistance, and Brent Bell for reading the manuscript.