J. Biochem. **139**, 821–829 (2006)
doi:10.1093/jb/mvj092

Association of Advanced Glycation End Products with A549 Cells, a Human Pulmonary Epithelial Cell Line, Is Mediated by a Receptor Distinct from the Scavenger Receptor Family and RAGE

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Received February 17, 2006; accepted February 24, 2006

Cellular interactions with advanced glycation end products (AGE)–modified proteins are known to induce several biological responses, not only endocytic uptake and degradation, but also the induction of cytokines and growth factors, combined responses that may be linked to the development of diabetic vascular complications. In this study we demonstrate that A549 cells, a human pulmonary epithelial cell line, possess a specific binding site for AGE-modified bovine serum albumin (AGE-BSA) (K_d = 27.8 nM), and additionally for EN-RAGE (extracellular newly identified RAGE binding protein) (K_d = 118 nM). Western blot and RT-PCR analysis showed that RAGE (receptor for AGE) is highly expressed on A549 cells, while the expression of other known AGE-receptors such as galectin-3 and SR-A (class A scavenger receptor), are below the level of detection. The binding of ^{125}I-AGE-BSA to these cells is inhibited by unlabeled AGE-BSA, but not by EN-RAGE. In contrast, the binding of ^{125}I-EN-RAGE is significantly inhibited by unlabeled EN-RAGE and soluble RAGE, but not by AGE-BSA. Our results indicate that A549 cells possess at least two binding sites, one specific for EN-RAGE and the other specific for AGE-BSA. The latter receptor on A549 cells is distinct from the scavenger receptor family and RAGE.

Key words: A549 cells, AGE, AGE-receptor, diabetes, EN-RAGE, glycation, RAGE, scavenger receptor.

Glucose and other reducing sugars, such as ribose and fructose, react with amino residues of proteins to form Schiff bases and Amadori products. Further incubation results in chemical rearrangements that convert these early products into irreversible derivatives termed advanced glycation end products (AGE). AGE are physiochemically characterized by their fluorescence, brown coloring and cross-linking, and biologically by their specific interactions with AGE-receptors. *In vivo* accumulation of AGE increases with aging and in age-related disorders such as diabetic complications (*1*–*3*) and atherosclerosis (*4*).

Cellular interactions with AGE-modified proteins induce several biological responses that may be involved in the development of diabetic vascular complications (*5*). These cellular interactions are thought to be mediated by AGE receptors such as SR-A (class A scavenger receptor types I and II) (*6*–*8*), CD36 (*9*), SR-BI (scavenger receptor class B type-I) (*10*), galectin-3 (*11*, *12*), LOX-1 (lectin-like Ox-LDL receptor-1) (*13*), HA-SR (hyaluronan scavenger receptor) (the same molecule as FEEL-1) (*14*, *15*) and RAGE (receptor for AGE) (*16*, *17*). Our previous studies identified SR-A, known to be a receptor for oxidized low density lipoprotein (ox-LDL) that mediates the endocytic uptake and lysosomal degradation of AGE-BSA by macrophages (*7*). CD36, which belongs to the class B scavenger receptor family, is recognized as an ox-LDL receptor and fatty acid transporter (*18*), and our recent experiments using Chinese hamster ovary (CHO) cells overexpressing CD36 demonstrated that CD36 also recognizes AGE-ligands (*9*). Furthermore, we demonstrated that scavenger receptor class B type-I (SR-BI), which is known to accelerate reverse cholesterol transport as a high density lipoprotein receptor, also acts as an AGE-receptor (*10*). Galectin-3, one of the lactose-binding lectin families found in basophils (*19*) and neutrophils (*20*, *21*), was identified as a component of the AGE receptor complex (*22*). Our previous study demonstrated that CHO cells overexpressing human galectin-3 exhibit specific binding not only for AGE-ligands, but also for acetylated LDL (acetyl-LDL) and ox-LDL, the authentic ligands of SR-A (*12*). LOX-1 was originally identified as a novel scavenger receptor for Ox-LDL, which is highly expressed on endothelial cells, and our subsequent study revealed that LOX-1 also serves as an AGE receptor (*13*). HA-SR was identified as an AGE receptor by collaboration between our group and Smedsrod et al. (*14*) by analyzing AGE receptors expressed on rat hepatic sinusoidal endothelial cells, and a subsequent study by Tamura *et al*. demonstrated that fasciclin EGF-like, laminin-type EGF-like, and link domain-containing scavenger receptor (FEEL-1) is identical to HA-SR and

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serves as an AGE-receptor (*15*). Taken together, these studies indicate that SR-A, CD-36, SR-BI and galectin-3 can also be categorized as AGE-receptors.

RAGE was originally isolated from bovine lung extracts (*16*) and belongs to the immunoglobulin superfamily; RAGE consists of three immunoglobulin domains, the V domain, C1 domain and C2 domain from the N-terminal (*17*), and is highly expressed by type II alveolar epithelial cells (*23*), neural cells and endothelial cells (*24*). Cellular interactions of AGE with RAGE are known to induce several cellular phenomena including the expression of vascular cell adhesion molecule-1 (VCAM-1) in endothelial cells (*25*), and cytokines in monocytes (*26*). Hori *et al*. (*27*) demonstrated that RAGE recognizes not only AGE but also amphoterin as a ligand, and that the interaction of amphoterin with RAGE induces nerve outgrowth. Furthermore, Hofmann *et al*. (*28*) recently identified EN-RAGE (extracellular newly identified RAGE binding protein) as a novel endogenous ligand for RAGE, and showed that EN-RAGE mediates the cellular activation of mononuclear phagocytes and lymphocytes (*28*). These studies emphasized the fact that RAGE might play a significant role in several biological systems. However, despite the ability of RAGE to recognize several ligands, the true physiological significance of RAGE as an AGE-receptor remains unclear because of its low binding rate for AGE-ligands.

A549 cells isolated from human lung carcinoma display a property closely similar to type II alveolar epithelial cells in that they also synthesize disaturated phosphatidylcholines (*29–31*) and secrete surfactants (*32–35*). Based on a previous report that RAGE is highly expressed in type II alveolar epithelial cells (*23*), we used A549 cells in the present study to determine whether AGE-ligands could be recognized by RAGE. The results provide evidence that, in addition to RAGE, A549 cells express another AGE-receptor that is distinct from other currently identified AGE-receptors such as galectin-3, SR-A, CD-36, SR-BI, LOX-1 and HA-SR, indicating the presence of a novel AGE-receptor on A549 cells.

MATERIALS AND METHODS

Chemicals—Bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO). Fetal bovine serum (FBS) and tissue culture medium were purchased from Gibco BRL (Gaithersburg, MD). Iodine-^{125} was obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). Horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG was acquired from Zymed (San Francisco, CA) and HRP-conjugated anti-mouse IgG was from Chemicon International Inc. (Temecula, CA). All other chemicals were of the highest grade available from commercial sources.

Ligand Preparation and Iodination—LDL (d = 1.019–1.063 g/ml) was isolated by sequential ultracentrifugation from fresh human plasma from normolipidemic subjects after overnight fasting, and dialyzed against 0.15 M NaCl and 1 mM EDTA (pH 7.4) (*36*). Acetyl-LDL was prepared by chemical modification of LDL with acetic anhydrate as described previously (*36*). Ox-LDL was prepared by incubating LDL with 5 μM of $CuSO_4$ for 20 h at 37°C as described previously (*36*). AGE-BSA was prepared as described previously (*37*). Briefly, 2.0 g of BSA and 3.0 g of glucose were dissolved in 10 ml of 0.5 M phosphate buffer (pH 7.4), incubated at 37°C for 40 weeks, and dialyzed against PBS. CML-BSA was prepared by incubating 175 mg of BSA with 0.15 M glyoxylic acid and 0.45 M $NaBH_3CN$ in 1 ml of 0.2 M phosphate buffer (pH 7.8) at 37°C for 24 h, followed by dialysis against PBS (*38*). Since AGE-BSA was prepared by incubating BSA with glucose, it possesses multi AGE structures. In contrast, CML-BSA was obtained with chemical carboxymethylation of lysine residues by glyoxylic acid and has only one kind of AGE structure on the BSA molecule. AGE-BSA and EN-RAGE were radiolabeled with ^{125}I using Iodo-Gen (Pierce) (*37*) and dialyzed against PBS. The specific activities of AGE-BSA and EN-RAGE were 963 and 2,225 cpm/ng, respectively.

Preparation of EN-RAGE—Recombinant EN-RAGE was prepared using an *Escherichia coli* expression system. Human EN-RAGE cDNA was obtained by two short length EN-RAGE fragments. Briefly, four oligonucleotides were synthesized based on the human EN-RAGE sequence (GenBank AF011757) (F1, 1–80; R1, 67–151; F2, 141–223; and R2, 214–282). In order to obtain two short-length EN-RAGE fragments, two sets (F1/R1 and F2/R2, respectively) were amplified by polymerase chain reaction (PCR). C-DNA derived from F1/R1 encompassed the first half of the coding region of human EN-RAGE, and that derived from F2/R2 covered the last half of the coding region of human EN-RAGE. By PCR using these two c-DNA with two primers (5:1–22 and 3: 254–282), a full-length EN-RAGE was established. The full-length human EN-RAGE was subcloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA) using the TA cloning method, and the sequences were confirmed to be identical to those of Hofmann *et al*. (*28*). EN-RAGE pCR2.1 was digested at the *Xba*1 and *Hin*d3 sites and then ligated into pMAL vector (New England BioLabs, Beverly MA) that expressed the maltose binding protein complex. The vector was transformed into *E. coli* and incubated overnight with 200 ml of LB medium containing 50 μg/ml of ampicillin (LB-A medium) at 37°C. A portion of the *E. coli* was further incubated with 1 liter of LB-A medium. When the absorbance at 660 nm of the medium reached 0.5, 10 ml of isopropyl-1-thio-β-D-galactopyranoside (100 mM) was added to the medium and the incubation was continued for a further 2 h, followed by centrifugation at 9,000 × g for 10 min at 4°C. The pellet was resuspended in washing buffer [20 mM Tris, 200 mM NaCl and 1 mM EDTA (pH 7.4)]. The suspension was sonicated, centrifuged at 7,000 × g for 10 min at 4°C, and the supernatant was filtered through a 0.45 μm disk filter and applied onto an amylose resin column (ϕ 1.5 × 8.3 cm, New England Biolabs). The column was washed with washing buffer, and then eluted with elution buffer (washing buffer containing 10 mM maltose). The eluted fraction was dialyzed against washing buffer and then digested overnight with Factor Xa (1 mg protein/2 μg Factor Xa) at room temperature. The digested sample was applied again to the amylose resin column, and purified recombinant EN-RAGE was collected as the non-adsorbed fraction. The purified EN-RAGE was confirmed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) to be a 10-kDa protein.

Preparation of Soluble RAGE—Recombinant soluble RAGE was prepared according to a previously reported

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method (*39*). Briefly, pMAL vector was cut at the *Bam*HI site, and a DNA coding for the extracellular domain of human RAGE was inserted into the vector, which was then transfected into *E. coli*. Soluble RAGE was purified from the *E. coli* lysate by the same method as described above for EN-RAGE. The purified soluble RAGE was confirmed by SDS-PAGE to be a 38-kDa protein.

Preparation of Recombinant Human Galectin-3—Recombinant human galectin-3 was prepared according to the method Hus *et al*. (*40*). Briefly, DNA coding for galectin-3 was inserted into pET vector (Novagen, Madison, WI) at the *Eco*RI site, followed by transformation into *E. coli*. Galectin-3 was purified from the *E. coli* lysate by galactose-conjugated Sepharose 4B column chromatography as a 32-kDa protein (*41*). A partial amino acid sequence of the N-terminal region revealed it to be a fusion protein of 12 of amino acids from the pET vector sequence and human galectin-3 (data not shown).

Antibodies—To make a polyclonal antibody against human galectin-3, 1.0 mg of galectin-3 in 50% Freund's complete adjuvant was injected intradermally into 20 skin sites of a rabbit, followed by five booster injections with 0.5 mg of galectin-3 in 50% Freund's incomplete adjuvant. Serum was obtained 10 days after the final immunization, the IgG was purified by protein G–conjugated affinity chromatography.

To prepare human anti-RAGE antibody, a RAGE specific peptide for intracellular lesion (E^{392}EPEAGESSTGGP^{404}) was designed from the human RAGE sequence (GenBank D28769) and conjugated with hemocyanin from keyhole limpet (RAGE-KLH). RAGE-KLH was used as the antigen to prepare the polyclonal anti-RAGE antibody. The immunization and purification were the same as for the anti–galectin-3 antibody.

The monoclonal anti–SR-A antibody (F8) was obtained by immunizing SR-knockout mice with recombinant human type I SR protein as an immunogen (*42*).

Cellular Assay—Unless otherwise stated, cell culture experiments were performed in 5% CO_2 at 37°C. A549 cells were obtained from RIKEN Cell Bank (Ibaragi, Japan) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. Differentiated THP-1 cells were obtained by incubating the cells with 200 nM phorbol 12-myristate 13-acetate (PMA) in RPMI 1649 medium containing 10% FBS for 5 days (*43*). For binding experiments, A549 cells (3×10^4) were seeded in a 24-well plate, and cultured at 37°C for 2 days in 1 ml of DMEM containing 10% FBS. The cells were washed twice with 1 ml of PBS, and the medium was replaced with 0.5 ml of KRH buffer [136 mM NaCl, 4.7 mM KCl, 1.25 mM $MgSO_4$, 1.25 mM $CaCl_2$, 20 mM HEPES, 0.1% glucose and 1% BSA (pH 7.4)] containing various concentrations of ^{125}I-AGE-BSA or ^{125}I-EN-RAGE in the presence or absence of 20-fold excess amounts of the unlabeled ligands. The cells were incubated for 90 min at 4°C, and washed once with 0.5 ml of ice-cold PBS containing 1% BSA and twice with ice-cold PBS. The cells were lysed for 30 min at 37°C with 0.5 ml of 0.1 N NaOH, and the cell-bound radioactivity was determined. Additionally the cellular protein concentration was determined using the bicinchoninic acid protein assay reagent (Pierce).

Immunoblotting—Cells were washed twice with PBS and lysed with 0.2 ml of 50 mM Tris-HCL (pH 7.4) containing 150 mM sodium chloride, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml pepstatin, 50 μg/ml leupeptin, 0.1 μg/ml aprotinin, 1 mM EDTA and 1% Triton X-100. The lysate was centrifuged at 10,000 × *g* for 15 min at 4°C. Supernatants (corresponding to 5 μg/lane) were subjected to SDS-PAGE and electrotransferred onto a nitrocellulose membrane. The membrane was blocked for 1 h at room temperature with 5% nonfat dry milk in 20 mM Tris-HCl (pH 7.4) containing 0.9% sodium chloride and 0.05% Tween 20 (buffer A), and washed three times with buffer A. The membrane was then incubated for 2 h at room temperature with the antibody to be tested (5 μg/ml of anti–galectin-3 antibody, 2 μg/ml of anti-RAGE antibody or the anti–human SR-A antibody) and washed three times with buffer A. Immunoreactive bands were detected by incubation for 1 h with a 1:5,000 dilution of HRP-conjugated goat secondary anti-rabbit IgG (Zymed Laboratories Inc., San Francisco, CA) in 5% nonfat dry milk in buffer A followed by washing three times with buffer A, with visualization by chemiluminescence (ECL™: Amersham).

Reverse Transcription PCR (RT-PCR) of RAGE—Total RNA was isolated by TRIzol (Life Technologies, Gaithersburg, MD), and reverse transcription was performed using oligo dT (Gibco BRL) with RNase H-free reverse transcriptase (Superscript II; Gibco BRL). Specific primers for PCR were designed from the human RAGE sequence as follows: 5′-CAATGAACAGGAATGGAAAG (sense) and 5′-TCCTCTTCCTCCTGGTTTT (antisense). PCR consisted of denaturation at 94°C for 10 min followed by a 30-cycle program of denaturation at 94°C for 30 s, annealing at 60°C for 60 s, and extension at 75°C for 90 s in a Gene Amp PCR system 9600 (Perkin Elmer-Cetus, Foster City, CA). The products were analyzed by agarose gel electrophoresis.

Preparation of RAGE Overexpressing CHO Cells—Human RAGE cDNA was obtained from a lung cDNA library by a PCR method (sense primer: 5′-CCAGGACCCTGGAAGGAAG, anti-sense primer: 5°-ACAATGATGATTAAACACCTGACACAT), and inserted into pCR3.1 vector (Invitrogen). The sequence and the orientation of the construct were confirmed by DNA sequencing, and the construct was then transfected into CHO cells by lipofectAMINE according to the protocol recommended by the manufacturer (Gibco BRL, Rockville, MD, USA). One day after transfection, the medium was changed to Ham's F-12 medium containing 10% fetal calf serum and 0.8 mg/ml of G418 (medium A). Upon continuous incubation for one week, colonies resistant to G418 were screened by immunoblotting using the polyclonal anti-human RAGE antibody and RT-PCR, and colonies positive for RAGE were identified. Three independent positive colonies were isolated and subjected to further screening, and one of these colonies was used in the present experiments as RAGE-transfected CHO cells (RAGE-CHO cells); two other RAGE-transfected CHO cells gave results similar to those obtained in the present study (data not shown). Unless otherwise stated, RAGE-CHO cells and wild CHO cells were cultured in culture dishes (100 mm in diameter) with 10 ml of medium A. CD36 overexpressing CHO cells (CD36-CHO cells) (*9*) were also used as a positive control for the uptake study.

Immunofluorescence Microscopy—The RAGE-CHO cells were cultured for 2 days in an ECM coated chamber slide

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(Asahi Techno Glass Corp. Japan). The cells were washed with PBS and fixed with 100% methanol for 1 min. The slide was blocked for 1 h at room temperature with 1% non-immune goat serum and 0.5% BSA in PBS containing 0.05% Tween 20 (buffer A), and then incubated for 1 h at room temperature with anti-RAGE antibody (10 μg/ml) and washed three times with buffer A. Primary antibody was detected after incubation for 1 h with fluorescence isothiocyanate-conjugated anti-rabbit IgG (Zymed Laboratories Inc., San Francisco, CA) in buffer A and washing three times with PBS. Nuclei were stained with a propidium iodide (PI) nucleic acid staining kit (Molecular Probes, Eugene, OR), mounted, and then observed under a confocal laser scanning microscope (FLUOVIEW, Olympus, Japan).

RESULTS

Expression of AGE-Receptors on A549 Cells—We performed immunoblotting analysis with antibodies against RAGE, galectin-3 and SR-A. The anti–galectin-3 antibody reacted significantly with recombinant human galectin-3 (32-kDa), whereas its reactivity with A549 cell lysates was below the detection level (Fig. 1A). Immunoblot analysis with the anti–SR-A antibody showed a positive band (approximately 70-kDa) from PMA-treated THP-1 cells, whereas no significant band was detected from the PMA-untreated THP-1 cells or A549 cells (Fig. 1B). In contrast, the anti-RAGE antibody revealed a positive band of approx. 46-kDa in A549 cells (Fig. 1C). In addition, RT-PCR analysis detected an 850 bp-band of RAGE (Fig. 1D). These results demonstrate that A549 cells express a significant level of RAGE, but minimal or no galectin-3 and SR-A.

Binding of ^{125}I-AGE-BSA and ^{125}I-EN-RAGE to A549 Cells—We determined the binding of ^{125}I-AGE-BSA to A549 cells. The total binding of ^{125}I-AGE-BSA was inhibited by a 20-fold excess of unlabeled AGE-BSA. The specific binding, obtained by subtracting the nonspecific binding from the total binding, showed a saturation curve, and Scatchard analysis of this specific binding demonstrated a binding site with an apparent dissociation constant (K_d) value of 1.97 ng/ml (27.8 nM) and maximal surface binding of 24.5 ng/mg cell protein (Fig. 2A). Figure 2B shows the dose-dependent binding of ^{125}I-EN-RAGE. Unlabeled EN-RAGE was competitive for the binding of ^{125}I-EN-RAGE. Scatchard analysis of this specific binding disclosed a binding site with an apparent K_d of 1.41 ng/ml (118 nM) and maximal surface binding of 92.1 ng/mg cell protein. These results indicate that A549 cells possess high-affinity binding sites for AGE-BSA and EN-RAGE. We also examined the interaction of CML-BSA, a known ligand for RAGE, with A549 cells. However, this ligand did not show specific binding to A549 cells (data not shown). Thus, we continued to focus on AGE and EN-RAGE (as ligands for AGE-receptors in A549 cells) in the subsequent experiments.

Effect of Various Ligands on the Binding of ^{125}I-AGE-BSA and ^{125}I-EN-RAGE to A549 Cells—At least seven AGE-receptors have been reported, including RAGE, galectin-3, SR-A, CD36, SR-BI, LOX-1 and HA-SR (*6–14*). Based on these reports, we examined the type of AGE receptor that plays a major role in A549 cells. The binding of ^{125}I-AGE-BSA was effectively replaced by unlabeled AGE-BSA (>80%), whereas neither acetyl-LDL nor ox-LDL, an effective ligand for SR-A, CD36, SR-BI, LOX-1 and HA-SR, showed any significant effect, indicating that the AGE receptor functioning on A549 cells is obviously different from known scavenger receptor families. Neither lactosylated-BSA, a ligand for galectin-3, nor the anti–galectin-3 antibody had any inhibitory effect on the binding of ^{125}I-AGE-BSA to A549 cells, while the addition of galectin-3 slightly enhanced the binding of ^{125}I-AGE-BSA (Fig. 3A). CML-BSA and EN-RAGE have been reported to be effective ligands for RAGE (*28*, *44*); however, neither of them had any effect on the binding of ^{125}I-AGE-BSA to A549 cells (Fig. 3A). Similarly, the binding was not affected in any manner by soluble RAGE (Fig. 3A).

Since AGE-BSA has an inhibitory effect on the binding of ^{125}I-EN-RAGE to RAGE (*28*), we examined the effects of several ligands on the binding of ^{125}I-EN-RAGE to A549 cells. The binding of ^{125}I-EN-RAGE was significantly replaced by unlabeled EN-RAGE, whereas AGE-BSA, CML-BSA, ox-LDL, acetyl-LDL, lactosylated-BSA,

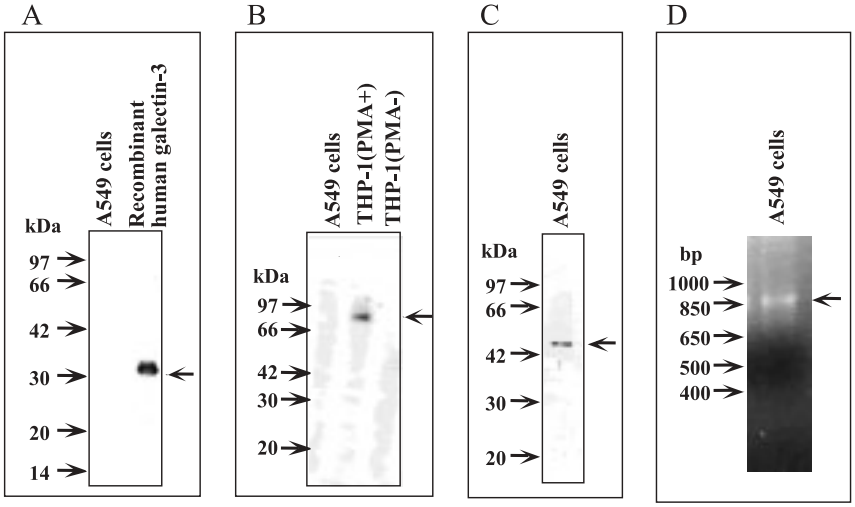

Fig. 1. **Detection of AGE-receptors on A549 cells by immunoblot analysis and RT-PCR.** (A) Cell lysates from A549 cells (1st lane 5μg) and recombinant human galectin-3 (2nd lane 0.01 μg) were subjected to 12.5% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with the anti–galectin-3 antibody. (B) Cell lysates from A549 cells (1st lane, 5μg), PMA-treated THP-1 cells (2nd lane, 5μg) and PMA-untreated THP-1 cells (3rd lane, 5μg) were subjected to 10% SDS-PAGE and immunoblotted with the anti–SR-A antibody. (C) Cell lysates from A549 cells (5 μg) were subjected to 12.5% SDS-PAGE and immunoblotted with the anti-RAGE antibody. (D) Total RNA was extracted from A549 cells and the RAGE cDNA was amplified by PCR for 30 cycles followed by analysis in a 1.0% agarose gel.

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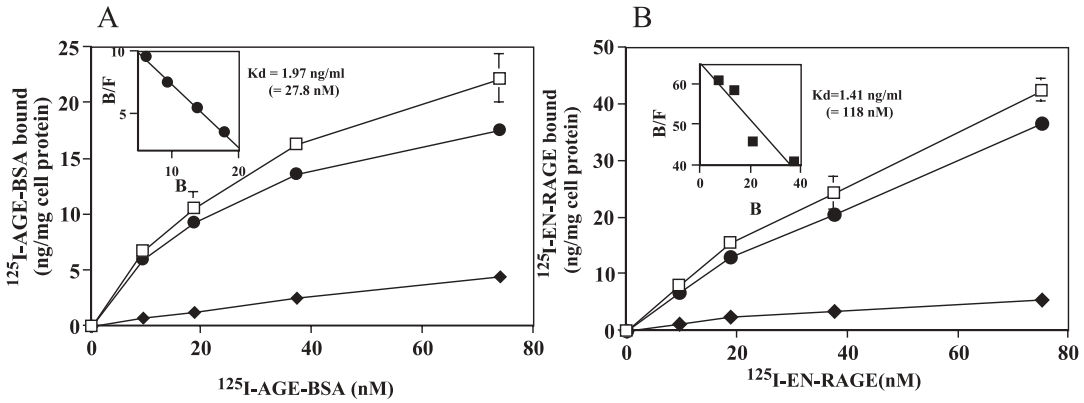

Fig. 2. **Binding of ^{125}I-AGE-BSA and ^{125}I-EN-RAGE to A549 cells.** Cells were incubated for 90 min at 4°C in 0.5 ml of KRH buffer with increasing concentrations of ^{125}I-AGE-BSA (A) or ^{125}I-EN-RAGE (B) in the presence (diamonds) or absence (squares) of 20-fold excess amounts of unlabeled ligands. The cells were then washed and lysed in 0.1 N NaOH, and cell-bound radioactivity was determined. The specific binding (circles) was obtained by subtraction of nonspecific binding (diamonds) from the total binding (squares). Inset, Scatchard analysis of the specific binding curve. Data are means ± SD.

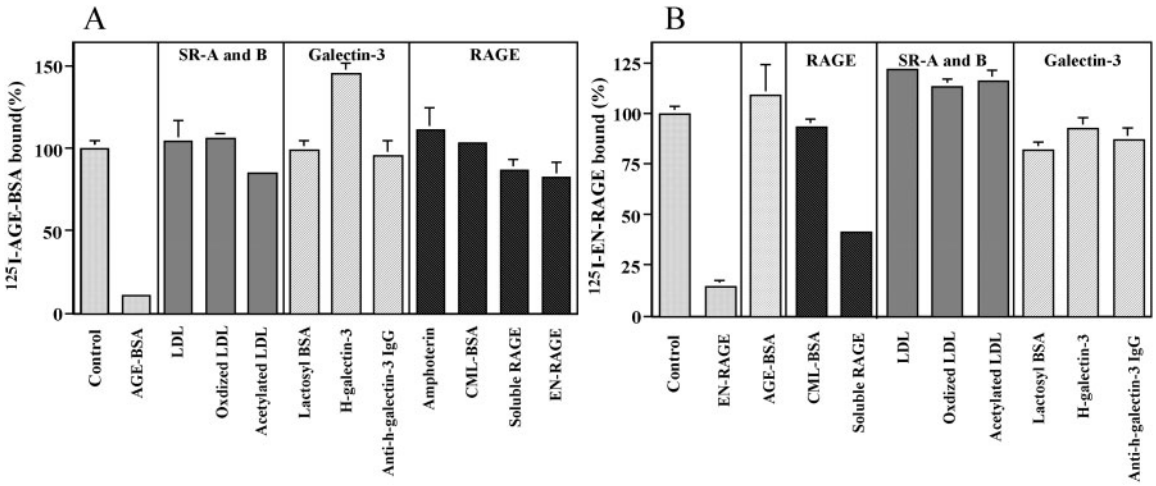

Fig. 3. **Effects of several ligands on the binding of ^{125}I-AGE-BSA and ^{125}I-EN-RAGE to A549 cells.** Cells were incubated at 4°C for 90 min in 0.5 ml of KRH buffer with 1.25 μg/ml of ^{125}I-AGE-BSA (A) or ^{125}I-EN-RAGE (B) in the absence (control) or presence of 50-fold excesses of unlabeled ligands. The values for 100% binding of ^{125}I-AGE-BSA (A) and ^{125}I-EN-RAGE (B) in the absence of unlabeled ligands were 47.6 and 45.1 ng/mg cell protein, respectively. Data are expressed as the percent of control and represent means ± SD.

galectin-3 and the anti–galectin-3 antibody had no inhibitory effect. In contrast, the binding of ^{125}I-EN-RAGE was significantly inhibited by soluble RAGE (>60%) (Fig. 3B). This result confirms that A549 cells possess RAGE and provides evidence that EN-RAGE is bound to A549 cells through RAGE.

These results regarding the ligand specificity of AGE-BSA and EN-RAGE suggest that A549 cells have different binding sites for these ligands. In order to test this theory we performed several cross-competitive experiments.

Cross-Competitive Effects of AGE-BSA and EN-RAGE on Their Binding to A549 Cells—The binding of ^{125}I-AGE-BSA to A549 cells was effectively inhibited by unlabeled AGE-BSA in a dose-dependent manner, whereas EN-RAGE had no effect on the binding (Fig. 4A). Under identical conditions, the presence of unlabeled EN-RAGE significantly competed with the binding of ^{125}I-EN-RAGE to A549 cells in a dose-dependent manner, whereas unlabeled AGE-BSA showed no such inhibitory effect (Fig. 4B). These results strongly suggest that while EN-RAGE binds to RAGE expressed on A549 cells, AGE-BSA is recognized by a receptor distinct from RAGE and other known AGE-receptors including galectin-3, SR-A, CD36 and SR-BI.

Overexpression of Human RAGE in CHO Cells—CHO cells overexpressing human RAGE (RAGE-CHO cells) were prepared as described under "MATERIALS AND METHODS." The expression of RAGE in these cells was confirmed by RT-PCR and immunoblot analysis. RT-PCR analysis detected an 1,408-bp band of RAGE), whereas no visible band was observed in wild cells (Fig. 5A). Immunoblot analysis with the anti-RAGE antibody revealed a positive band (approximately 46 kDa) from RAGE-CHO cells, whereas no significant band was detected from wild CHO cells (Fig. 5B).

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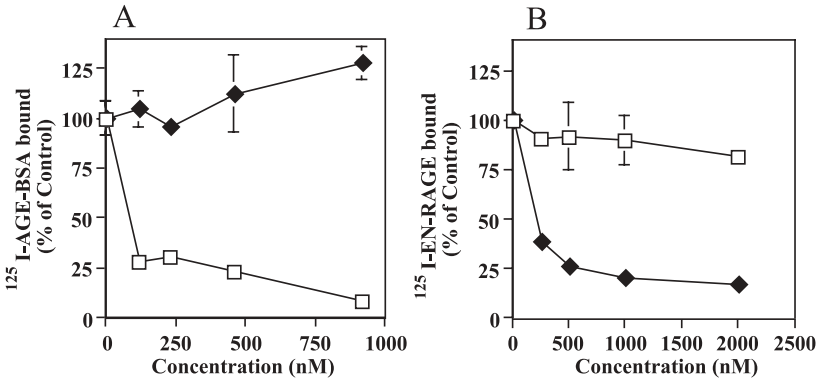

Fig. 4. **Cross-competitive effects of AGE-BSA and EN-RAGE on their binding to A549 cells.** Cells were incubated at 4°C for 90 min in 0.5 ml of KRH buffer with 1.25 μg/ml of ^{125}I-AGE-BSA (A) or ^{125}I-EN-RAGE (B) in the presence of the indicated concentrations of unlabeled AGE-BSA (squares) or unlabeled EN-RAGE (diamonds). The values for 100% binding of ^{125}I-AGE-BSA (A) and ^{125}I-EN-RAGE (B) in the absence of unlabeled ligands were 37.5 and 64.8 ng/mg cell protein, respectively. Data are expressed as the percent of control and represent means ± SD.

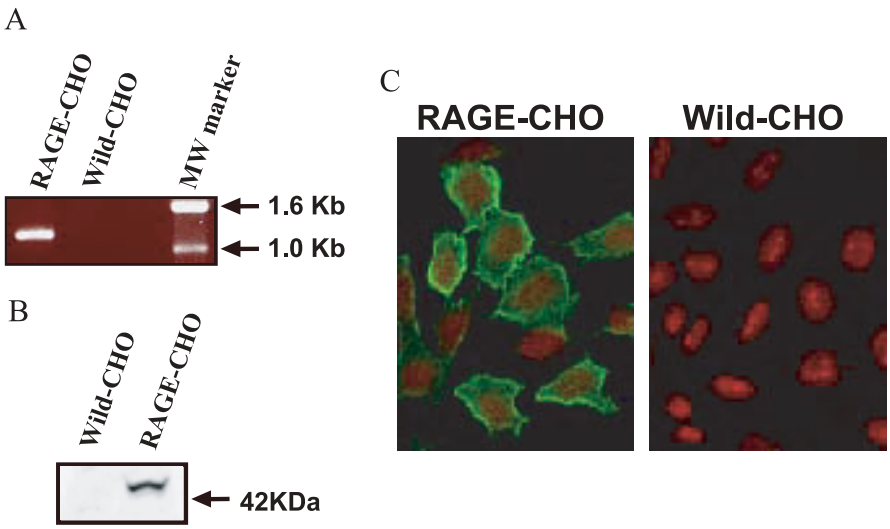

Fig. 5. **Expression of RAGE on RAGE-CHO cells.** (A) Total RNA was extracted from RAGE-CHO or wild-CHO cells, and RAGE cDNA was amplified by PCR for 30 cycles followed by analysis in a 1.0% agarose gel. (B) Cell lysates from RAGE-CHO or wild-CHO cells (10 μg/lane) were subjected to 12.5% SDS–polyacrylamide gel electrophoresis and immunoblotted with the anti-RAGE antibody as described under "MATERIALS AND METHODS." (C) RAGE-CHO or wild-CHO cells were cultured in chamber slides and fixed with 100% methanol for 1 min, treated with anti-RAGE antibody and by fluorescence isothiocyanate–conjugated anti-rabbit antibody to visualize RAGE. Nuclei were also visualized by PI staining.

To confirm the localization of RAGE, we performed an indirect immunofluorescence study. RAGE was found to be expressed on the cell surface of RAGE-CHO cells, but not on wild-type CHO cells (Fig. 5C).

Endocytic Uptake of ^{125}I-AGE-BSA by RAGE- and CD36-CHO Cells—We determined the endocytic uptake of ^{125}I-AGE-BSA by RAGE-CHO cells at 37°C. Since we previously demonstrated that CD36 overexpressing CHO cells (CD36-CHO) actively recognize AGE-BSA (*9*), we used CD36-CHO cells for a positive control in the present study. As shown in Fig. 6A, the level of specific cell association of ^{125}I-AGE-BSA with CD36-CHO cells increased in a dose-dependent manner and was competitively eliminated almost completely by a 20-fold excess of unlabeled AGE-BSA. In sharp contrast, RAGE-CHO cells did not associate ^{125}I-AGE-BSA at all under the same conditions (Fig. 6B). Furthermore, wild-CHO cells also demonstrated no endocytic association of ^{125}I-AGE-BSA (Fig. 6C).

DISCUSSION

We previously demonstrated that SR-A and galectin-3 act as AGE-receptors (*6–8*, *12*). Recent studies further showed that CD36 (*9*) and SR-BI (*10*), members of the class B scavenger receptor family, LOX-1 (*13*) and HA-SR (*14*) also serve as AGE-receptors. Interactions between AGE-ligands and AGE-receptors are known to induce several phenomena, including the stimulation of signal transduction pathways, activation of transcription factors and changes in cellular function. For example the P42^{MAP}-kinase pathway in the renal tubular cell line LLC-PK$_1$ is stimulated by AGE-ligands, resulting in the activation of the downstream target activator protein-1 (*45*). Interactions between AGE-ligands and AGE-receptors initiate many biological responses such as the induction of oxidative stress on endothelial cells *via* NF-kB (*46*), and the chemotaxis of mononuclear phagocytes (*28*) and rabbit smooth muscle cells (*47*). These findings suggest a potential link between the AGE-ligand and AGE-receptor system and basic cellular functions in vivo. Therefore, we speculate that AGE-receptor(s) expressed on pulmonary endothelial cells may affect their functions.

In the present study, we provide information regarding two new aspects of AGE-receptors. Firstly, A549 cells highly express RAGE (Fig. 1, C and D), which recognizes EN-RAGE as a ligand (Fig. 2B) but not CML-BSA. Second, A549 cells possess the binding site for AGE-modified BSA (Fig. 2A); however, this binding is not inhibited by CML-BSA and EN-RAGE, which are reported to be effective as ligands for RAGE (Fig. 3A). Therefore, it is likely that the binding site or AGE-receptor expressed on A549 cells is novel, since it is distinct from other known

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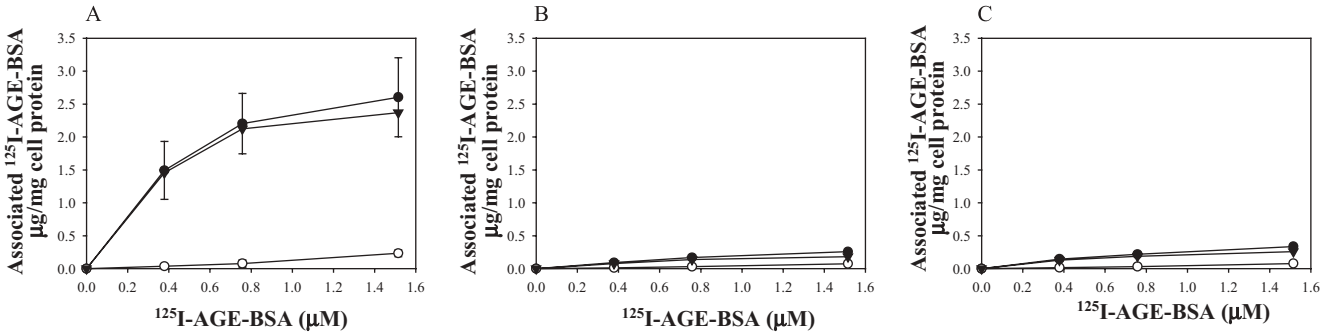

Fig. 6. **Endocytic uptake of ^{125}I-AGE-BSA by RAGE- and CD36-CHO cells.** CD36-CHO (A), RAGE-CHO (B) and Wild-CHO (C) cells were incubated for 5 h at 37°C with various concentrations of ^{125}I-AGE-BSA in the presence (open circles) or absence (solid circles) of 20-fold excess of unlabeled AGE-BSA. Specific association (inverted triangles) was determined by subtracting nonspecific association from total association as described under "MATERIALS AND METHODS." Data are means ± SD.

AGE-receptors such as RAGE, galectin-3, SR-A, CD36 and SR-BI. There are four possibilities that should be taken into consideration in explaining why no AGE/RAGE interaction could be detected on A549 cells. First, as described in the Introduction, the binding affinity between RAGE and AGE-proteins is low, and our assay system was not able to detect their binding. In support of this, Schmidt *et al*. demonstrated that 10 μg of purified RAGE maximally binds 1.5 fmol ^{125}I-AGE-BSA (*16*), demonstrating a binding fraction between RAGE molecules and AGE-BSA molecules estimated to be 200,000:1. Therefore, the detection of an AGE-ligands/RAGE interaction might have been below the detection limit in our assay system. To confirm this possibility, we then prepared RAGE overexpressing CHO cells and measured the ligand activity of AGE-BSA to RAGE. Although AGE-BSA was significantly recognized by CD36-CHO cells (Fig. 6A), the RAGE-CHO cells did not uptake AGE-BSA as a ligand (Fig. 6B), demonstrating that the binding affinity between RAGE and AGE-proteins is below the detection limit in the present study. Second, since galectin-3, which lacks a transmembrane region, functions as an AGE-receptor by forming a complex with OST-48 and 80K-H (*11*), it is possible that cellular cofactors might be needed for ligands to bind properly to RAGE. Third, although our assay system previously demonstrated that scavenger receptor family members such as SR-A, CD36, SR-BI, LOX-1 and HA-SR significantly recognize AGE-BSA, we could not detect an interaction between AGE-BSA and RAGE or soluble RAGE. AGE-proteins are prepared in vitro by different research groups with different protocols. When Schmidt *et al.* reported RAGE as one of the AGE receptors, they prepared AGE-BSA by incubating BSA with glucose-6-phosphate (*16*), whereas we used glucose. Westwood *et al*. prepared AGE-BSA by incubating BSA with methylglyoxal, and demonstrated that their AGE-BSA was recognized by murine P388D$_1$ macrophages (*48*). Takeuchi *et al.* used acetaldehyde for the production of AGE-BSA, and demonstrated that their AGE-BSA showed toxicity to cortical neuronal cells (*49*). Therefore, it is likely that the ligand activity of glucose-derived AGE-BSA for RAGE might be different from that of glucose-6-phosphate–derived AGE-BSA. Finally, although RAGE expressed on A549 cells possesses an active binding site for EN-RAGE, the binding site for AGE-ligands might have been in an inactive form. The binding of ^{125}I-EN-RAGE to A549 cells was significantly inhibited by soluble RAGE and unlabeled EN-RAGE (Fig. 3B), indicating that RAGE expressed on A549 cells possesses an active binding site for EN-RAGE. In contrast, RAGE expressed on A549 cells failed to recognize AGE-BSA or CML-BSA as effective ligands. Although RAGE consists of three domains, the V domain, C1 domain and C2 domain from the N-terminal (*17*), its ligand binding domain has not yet been clarified. A recent study by Kislinger *et al*. (*44*) demonstrated that a CML-protein adduct is recognized by a recombinant V domain of RAGE, whereas neither of the recombinant C1 domain nor C2 domain showed any such an effect, indicating that the CML binding site of RAGE might occur through the V domain. RAGE is known to exhibit polymorphisms (*50*, *51*). When human RAGE DNA from patients with type II diabetes was analyzed by PCR and SSCP (single-strand conformation polymorphism), four different mutations were found, including Gly82Ser, Thr187Pro, Gly329Arg and Arg389Gln, but these mutations did not differ from those found in normal subjects (*45*). On the other hand, Poirier *et al*. (*46*) identified five independent polymorphisms including Cys-1152Ala in RAGE promoter region, Thr-388Ala in the promoter region, Ala2Ala in exon 1, Gly82Ser in exon 3 and Gly+196Ala in the downstream region of the RAGE gene, among which the Cys-1152Ala mutation showed a correlation with nephropathy (*46*). It is not known whether these RAGE polymorphisms influence the function of RAGE as an AGE-receptor.

In conclusion, the present study provides the evidence that A549 cells express novel AGE-receptor(s), which may play an important role in the cellular interaction with AGE-modified proteins.

This work was supported in part by Grants-in-Aid for Scientific Research (16790511 to Ryoji Nagai) from the Ministry of Education, Science, Sports and Culture of Japan. We are grateful to Norma Frizzell of the Department of Chemistry and Biochemistry, University of South Carolina, USA, for careful reading and editing of the manuscript.

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