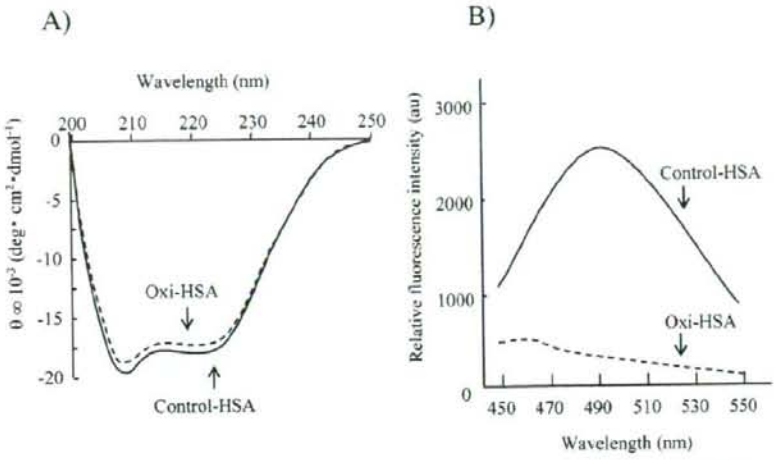

Fig. 2. Far-UV CD spectra (A) and relative fluorescence intensity of hydrophobicity using bis-ANS (B) of control-HSA (—) and oxi-HSA (···). Data are mean ± SD.

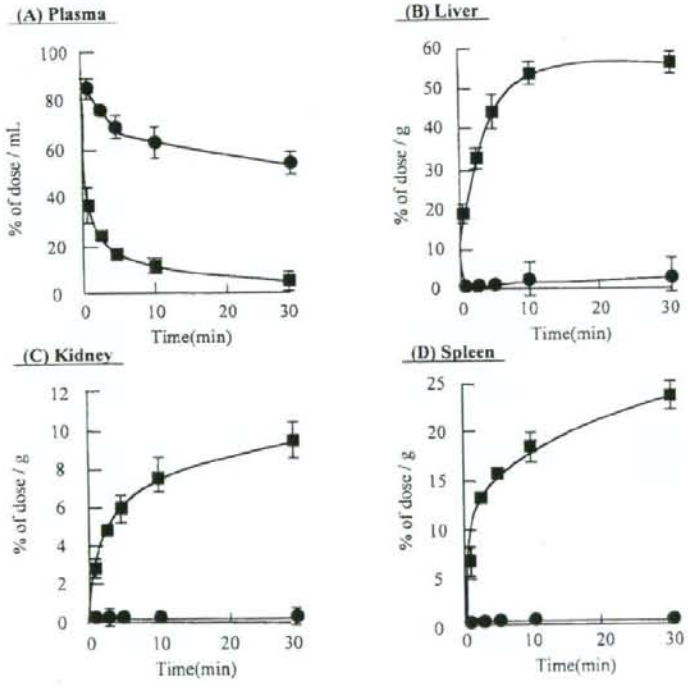

Fig. 3. Plasma and organ levels of ^{111}In-labeled control-HSA (●) and oxi-HSA (■) after i.v. administration to mice. Data are mean ± SD.

shown to have an extremely short half-life in circulating blood.[20] As one of reasons for the formation of modified albumin *in vivo*, free radical species have been found to induce conformational changes, degradation and aggregation of proteins in several diseases.[21] In uremic patients, AOPP are produced by HOCl-modified albumin cross-linking *in vivo*. Further, AOPP can act as mediators of oxidative stress, and they have

been implicated in the immune dysregulation associated with chronic uremia.[6,7] However, the mechanisms by which AOPP is degraded or eliminated from circulating blood remain unclear. Therefore, in the present study, we used CT-treated HSA (oxi-HSA) to examine the mechanisms involved in elimination of AOPP.

The AOPP and dityrosine content of oxi-HSA were similar to those of proteins from uremic plasma (**Table 1**). In the Western blot analysis, oxi-HSA was mostly present in the form of multimolecular aggregates, probably resulting from dityrosine cross-linking and/or disulfide bridges (**Fig. 1**). Further, the amino acid analysis indicates that oxidation of HSA induced significant modification of tyrosine and basic amino acid residues such as lysine and arginine (**Table 2**). These results suggest that the modifications of those amino acid residues are responsible for the conformational changes observed in HSA (**Fig. 2**).

The pharmacokinetic analysis consistently showed that oxi-HSA left the circulation rapidly. The organ distribution 30 min after the intravenous injection was 51% for the liver, 23% for the spleen, and 9% for the kidney, suggesting that the liver was the main route for plasma clearance (**Fig. 3**). Since many of the scavenger receptors are expressed in sinusoidal endothelial cells and Kupffer cells in the liver,[22,23] plural scavenger receptors may be responsible for the hepatic uptake of oxi-HSA. In general, most scavenger receptors can bind a variety of polyanionic ligands, including negatively charged albumins.[24,25] In some diseases including diabetes, AGE-albumins have well known as major negatively charged proteins in plasma. AGE-albumins are nonenzymatically glycosylated proteins that accumulate in vascular tissue with aging and accumulate at accelerated rates in diabetic patients. Schmidt *et al.*[22] reported that AGE-albumins plasma clearance in mice was rapid, with 70% of the injected dose removed from the circulation within 5 min. In their study, liver was also the main route for plasma clearance of AGE-albumin. Their data are very similar to the present findings for oxi-HSA. Interestingly, oxi-HSA has a negatively charged molecule due to the modifications of basic amino acid residues in structural analysis (**Table 2**). Further, oxi-HSA has known to contain a structural motif of AGE-proteins as a result of oxidation.[6,7] Therefore, oxi-HSA are possible to be distributed for liver nonparenchymal cells (endothelial and Kupffer cells) as same as those of AGE-albumins. However, the evidence for scavenger receptor involved in the elimination of modified albumin, including AGE-albumin and oxi-HSA, on liver nonparenchymal cells has been rarely found.[26,27] Unfortunately, we were also unable to clarify the receptor responsible for hepatic uptake in this study, but we should examine the elimination mechanism of oxi-HSA by using cell line highly expressed scavenger receptors in the future.

On the other hands, scavenger receptors are expressed not only on liver nonparenchymal cells but also various macrophages. Given the fact that the organ distribution of oxi-HSA was 23% for the spleen, oxi-HSA may be also taken up by macrophage of spleen via scavenger receptor-mediated endocytosis. Macrophage has known to express highly SR-A, one of the scavenger receptor.[8] To examine the relationship between oxi-HSA and SR-A in macrophage, we used the macrophage-derived cell line RAW 264.7. Significant amounts of ^{125}I-oxi-HSA were associated with these cells and underwent endocytic degradation within the cells, whereas no such findings were observed for control-HSA (**Fig. 4**). Thus, SR-A may be involved in the spleen clearance of oxi-HSA.

Table 3. Uptake clearance of control- and oxi-HSA labeled with ^{111}In after i.v. administration to mice.

(μL/hr)	Liver	Kidney	Spleen
Control-HSA	24 ± 4.2	48 ± 5.3	32 ± 2.7
Oxi-HSA	5058 ± 341.6*	1188 ± 208.2*	2118 ± 322.1*

*Significantly different ($P < 0.01$) from control-HSA.
Data are mean ± SD.

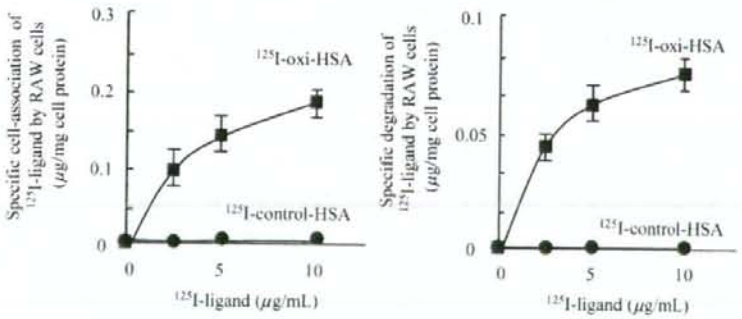

Fig. 4. Endocytic uptake of ^{125}I-labeled control-HSA (●) or oxi-HSA (■) by RAW 264.7 cells. Data are mean ± SD.

In summary, the kinetics of chloramine-T-treated HSA (oxi-HSA) are similar to those of AOPP in uremic patients. Oxi-HSA left the circulation rapidly and accumulated in the liver, spleen and kidney. The liver and spleen appear to play particularly important roles in elimination of AOPP.

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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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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

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′-TCCTC-TTCCTCCTGGTTTT (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′-CCAGGACC-CTGGAAGGAAG, anti-sense primer: 5′-ACAATGATGAT-TAAACACCTGACACAT), 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

(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,

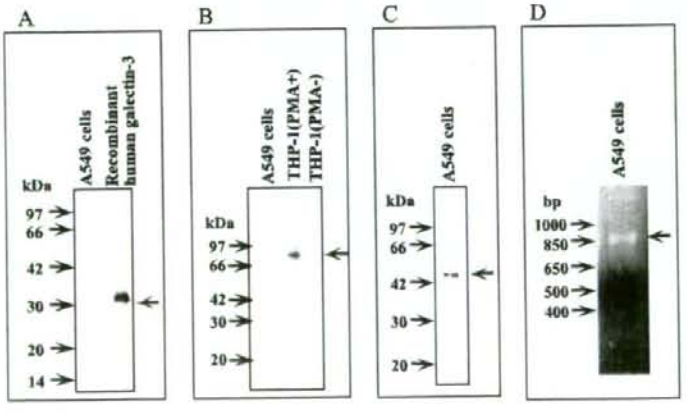

Fig. 1. **Detection of AGE-receptors on A549 cells by immunoblot analysis and RT-PCR.** (A) Cell lysates from A549 cells (1st lane 5μg) and recombinant human galectin-3 (2nd lane 0.01 μg) were subjected to 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with the anti-galectin-3 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.

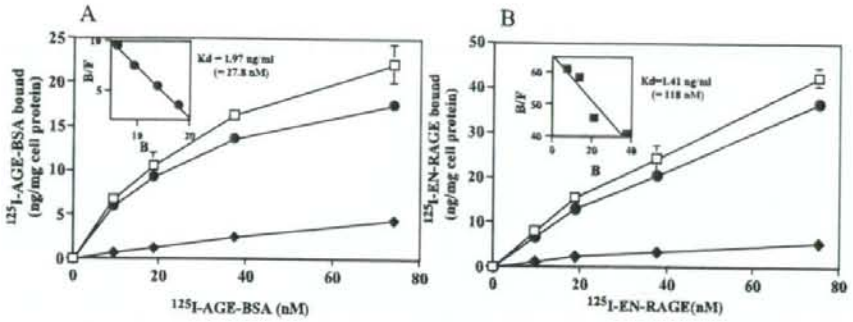

Fig. 2. **Binding of ^{125}I-AGE-BSA and ^{125}I-EN-RAGE to A549 cells.** Cells were incubated for 90 min at 4°C in 0.5 ml of KRH buffer with increasing concentrations of ^{125}I-AGE-BSA (A) or ^{125}I-EN-RAGE (B) in the presence (diamonds) or absence (squares) of 20-fold excess amounts of unlabeled 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.

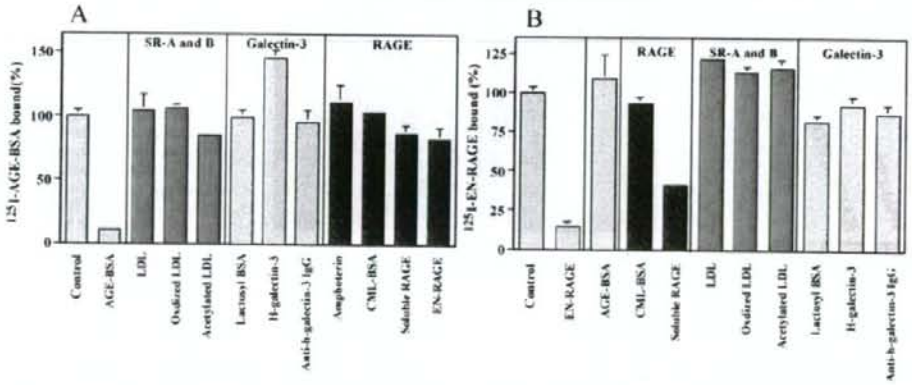

Fig. 3. **Effects of several ligands on the binding of ^{125}I-AGE-BSA and ^{125}I-EN-RAGE to A549 cells.** Cells were incubated at 4°C for 90 min in 0.5 ml of KRH buffer with 1.25 μg/ml of ^{125}I-AGE-BSA (A) or ^{125}I-EN-RAGE (B) in the absence (control) or presence of 50-fold excesses of unlabeled 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).

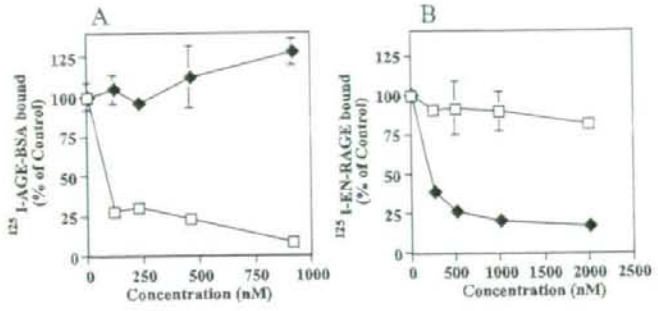

Fig. 4. **Cross-competitive effects of AGE-BSA and EN-RAGE on their binding to A549 cells.** Cells were incubated at 4°C for 90 min in 0.5 ml of KRH buffer with 1.25 μg/ml of ^{125}I-AGE-BSA (A) or ^{125}I-EN-RAGE (B) in the presence of the indicated concentrations of unlabeled AGE-BSA (squares) or unlabeled 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.

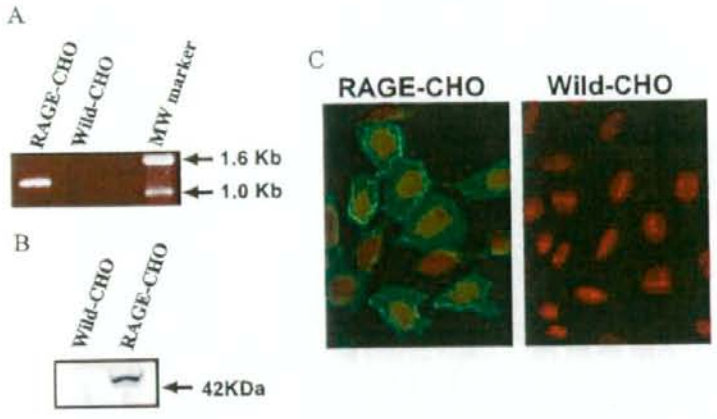

Fig. 5. **Expression of RAGE on RAGE-CHO cells.** (A) Total RNA was extracted from RAGE-CHO or wild-CHO cells, and RAGE cDNA was amplified by PCR for 30 cycles followed by analysis in a 1.0% agarose gel. (B) Cell lysates from RAGE-CHO or wild-CHO cells (10 μg/lane) were subjected to 12.5% SDS-polyacrylamide 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

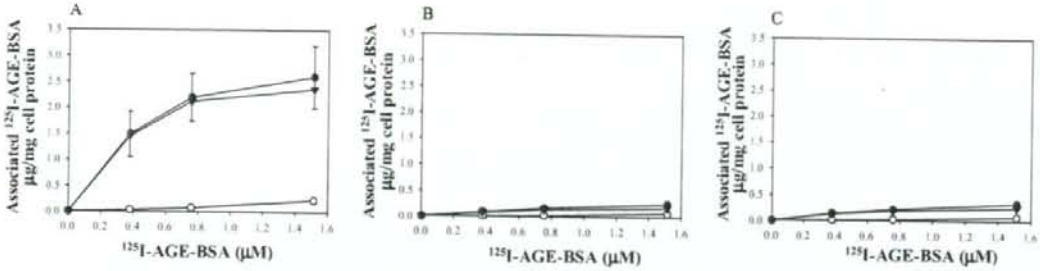

Fig. 6. **Endocytic uptake of ^{125}I-AGE-BSA by RAGE- and CD36-CHO cells.** CD36-CHO (A), RAGE-CHO (B) and Wild-CHO (C) cells were incubated for 5 h at 37°C with various concentrations of ^{125}I-AGE-BSA in the presence (open circles) or absence (solid circles) of 20-fold excess of unlabeled AGE-BSA. 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.

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Albumin Rescues Ocular Epithelial Cells from Cell Death in Dry Eye

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ABSTRACT *Purpose*: Because autologous serum is useful for the treatment of severe dry eye, serum components may be a potential candidate for the treatment of dry eye. Serum albumin is abundantly contained in human serum and plays many physiologic roles. We investigated the efficacy of serum albumin in a dry eye animal model. *Methods*: Sprague-Dawley rats were used to make dry eye model rats according to a previous study. The central region of the corneal epithelium was scraped mechanically, and the rats were placed in a desiccation room (temperature, 23 ± 2°C; humidity, 28 ± 2%; air flow, 2–4 m/s) for 12 hr. During desiccation, one eye of each rat was treated with human serum albumin eye drops, and the other eye was given a drop of phosphate buffered saline (PBS). Human corneal and conjunctival cell lines were used to investigate suppression effect of albumin on apoptosis induced by addition of apoptosis inducers or serum deprivation, respectively. *Results*: The erosion area was increased by 12 hr of desiccation. Albumin treatment decreased the area of erosion compared with PBS treatment. Apoptosis suppression assay using cell lines revealed that caspase-3 activation induced by serum deprivation and DNA fragmentation induced by addition of apoptosis inducers were dose-dependently suppressed by albumin. *Conclusions*: Albumin showed a therapeutic effect in dry eye model rats. This efficacy may be related to the suppression of apoptosis by albumin.

KEYWORDS albumin; apoptosis; conjunctiva; cornea; dry eye

INTRODUCTION

Severe dry eye results not only from dryness of the ocular surface but also from the lack of tear components, which are essential for maintaining the ocular surface.[1–5] Artificial tears are useful for the treatment of dry eye, but the efficiency of the components included in artificial tears is not comparable with that of human tears. In a previous study, we used autologous serum as a substitute for tears for the treatment of severe dry eye and observed dramatic improvement of the ocular surface.[6] The efficient therapeutic effects of serum in cases of severe dry eye also suggest that the components of serum that are essential for maintaining a viable ocular surface may be the same or similar to those of tears.[7] A comparative analysis revealed that serum components were similar to tear components.[8]

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Human serum albumin (HSA) is monomeric single-chain protein synthesized and secreted by liver cells. HSA is constituted from 585 amino acids and does not have any prosthetic groups.[9,10] Its molecular weight is 66.5 kDa, and the ratio of HSA in serum proteins is approximately 50%.[8] Because HSA is a remarkably stable protein, it is distributed widely in body fluids. Many techniques used to investigate protein composition of human tears[11–13] revealed that HSA was also distributed in tears, where the ratio of albumin in tears was approximately 1%.[8]

In our previous study, we used 50 mg/ml albumin as eye drops for patients with Sjögren syndrome with severe dry eye.[14] The patients showed significant improvement in fluorescein and rose bengal scores, but not in tear break-up time and subjective symptoms. In this study, we investigate the effect of albumin as artificial tears for dry eye *in vivo* and *in vitro*.

MATERIALS AND METHODS

Animals

Male 8-week-old Sprague-Dawley rats (n = 12 in each experiment) were purchased from Tokyo Laboratory Animal Science Co., Ltd. (Tokyo, Japan). They were quarantined and acclimatized before the experiments for a 1-week period under standard conditions: room temperature 23 ± 2°C, relative humidity 60 ± 10%, an alternating 12-hr light-dark cycle (8 am. to 8 pm.), with water and food available *ad libitum*. All procedures were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Dry Eye Model Rat

The dry eye model rat experiment was performed according to a previous study.[15] The rats were anesthetized by intramuscular injection of an anesthesia cocktail, and the central region of the corneal epithelium (0.4 mm^2) was scraped mechanically with an ophthalmic surgical blade. After scraping, the rats were placed in a desiccation room, with room temperature of 23 ± 2°C, relative humidity of 28% ± 2%, and constant air flow (2–4 m/s), and maintained for 12 hr. During desiccation, one eye of each rat was treated with HSA (Wako Pure Chemical Industries, Ltd., Osaka, Japan) dissolved in PBS and the other eye was given a drop of PBS as the control. The concentration of HSA was 1, 5, and 10 mg/ml, respectively. The range of HSA concentration in eye drop was based on the following reasons: In a previous study, we used autologus diluted serum (20% serum) as a substitute for tears for the treatment of dry eye.[7] The concentration of albumin in diluted serum is approximately 7 to 11 mg/ml. Ten microliters of eye drops was administered every hour for 12 hr.

The damaged areas were photographed immediately and 12 hr after scraping by applying a fluorescein solution under cobalt-blue light. The stained area (epithelial erosion) was digitized with an optical scanner and quantified with image-analysis software (NIH Image, distributed by Wayne Rasband, National Institutes of Health, Bethesda, MD, USA).

Apoptosis Assay

Human conjunctival cell line CCL-20.2 (CCL) was obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were grown to 70–80% confluence in 75-cm^2 flasks with Medium 199 (Gibco BRL Life Technologies, Rockville, MD, USA) containing 10% fetal calf serum (FCS; Gibco BRL Life Technologies). The medium was removed, and cells were cultured for 24 hr in serum- free Medium 199 with or without HSA. The cells were harvested and solubilized with 50 mM HEPES buffer (pH 7.4) containing 0.1% {3-[(3-Cholamidopropyl)-dimethylammonio}-1-propanesulfonate (CHAPS), 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), and 0.1 mM ethylenediaminetetraacetic acid (EDTA) for 5 min in an ice bath. They were then centrifuged at 10,000 × *g* for 10 min at 4°C to obtain the supernatants. The caspase activities of cell extracts were measured using artificial fluorescent substrates (DEVD-AFC; Enzyme System Products, Livermore, CA, USA) with a fluorescent microplate reader (ARVO SX; PerkinElmer Life Sciences Japan Co., Ltd., Tokyo, Japan).[16]

Cell extracts were mixed with 50 mM HEPES buffer (pH 7.4) containing 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 0.1 mM EDTA, and 10% glycerol in 96-well plate. Enzyme reaction was started by addition of 0.1 mM artificial substrate to well. Fluorescence was measured immediately and after incubation for 1 hr at 37°C (excitation wavelength, 400 nm; emission wavelength, 505 nm).

Human corneal epithelial cell line CEPI-17-CL4 (CEPI) was kindly provided by Dr. Kuwahara (Alcon Laboratories, Fort Worth, TX, USA). These cells were immortalized by infection with a recombinant SV40-retrovirus vector containing the Bg1I-HpaI fragment of SV40 T-antigen[17] and express an extensive array of

cytokines, growth factors, and metabolic enzymes that resemble the original tissue.[18] CEPI was cultured to 70–80% confluence in 96-well plate with keratinocyte basal medium 2 (KBM-2; Clonetics Corp., San Diego, CA, USA). The cells were treated with apoptosis inducers (anti-Fas antibody and etoposide) in the presence or absence of 5 mg/ml HSA. DNA fragmentation was measured by using Cell Death Detection ELISA (Roche Diagnostics Corporation, Indianapolis, IN, USA). This assay was conducted according to the manufacturer's protocol. Briefly, DNA fragments were bound to peroxidase-labeled anti-DNA antibody, and the production of antibody-DNA fragment complex was measured photometrically using 2,2′-Azinobis(3-ethylbenzothiazolin-6-sulfonate) (ABTS) as the substrate.

RESULTS

Dry Eye Model Rat

The fluorescein-staining area in Figure 1A was a scraped area. In the control group, the scraped region of the corneal epithelium gradually diminished and disappeared within 12 hr.[15] In the dry eye group, the scraped region was clearly aggravated after 12 hr in the desiccation chamber (Fig. 1B).

To investigate the effect of HSA on corneal epithelial defects induced by desiccation, clinical observations were done after 12 hr of desiccation. HSA treatment decreased the area (Fig. 1C), whereas PBS treatment only had a mild effect (Fig. 1D) compared with no treatment. The effect of HSA is shown in Figure 2. Albumin eye drop decreased the fluorescein-stained areas. Significant decreases in the fluorescein-stained areas were observed in the 5 mg/ml eye drop group compared with PBS control ($p < 0.01$).

Apoptosis Assay

It was shown that apoptosis was induced in the conjunctiva[19] and corneal epithelium[20] in dry eye, although the mechanism involved is still unclear. Previously, our study showed that aggravation of the scraped

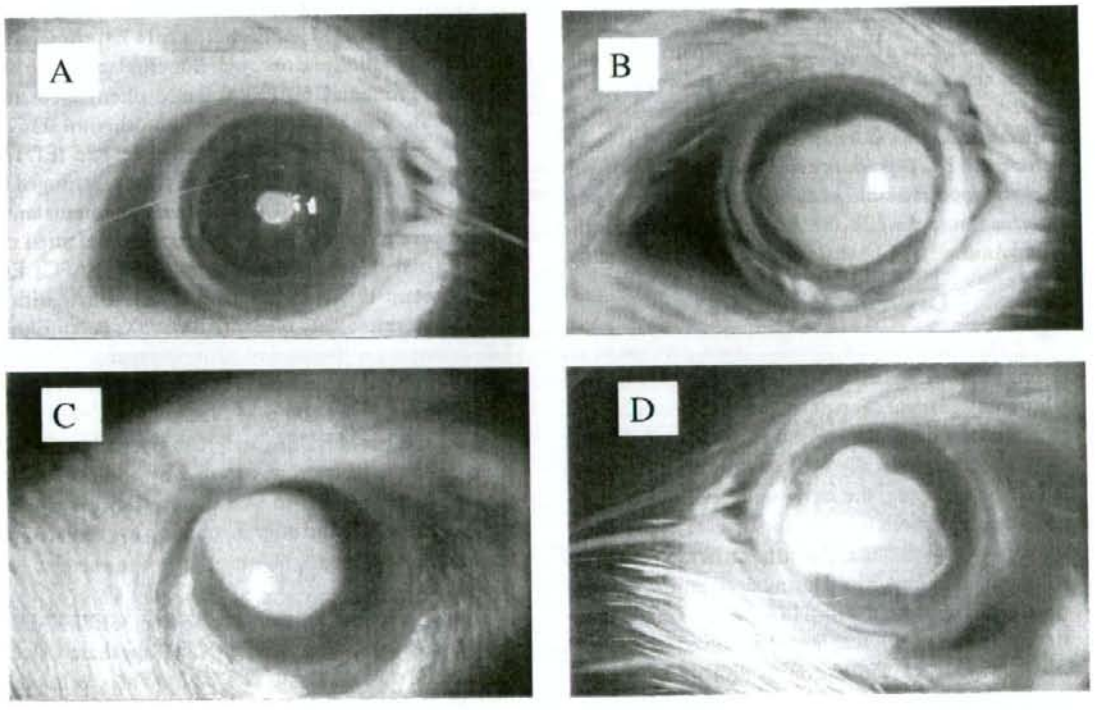

FIGURE 1 Fluorescein staining of corneal epithelial erosion induced by ocular surface desiccation. The damaged areas were photographed by applying fluorescein solution under cobalt-blue light. (A) Zero hours after scraping; (B) 12 hr after scraping with no treatment; (C) 12 hr after scraping with 5 mg/ml HSA treatment; (D) 12 hr after scraping with PBS treatment.

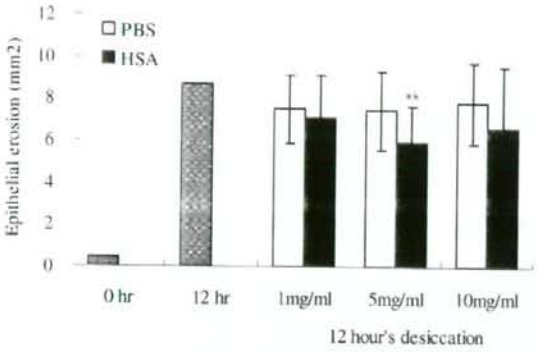

FIGURE 2 The size of epithelial erosion before treatment, without treatment, and after treatment with three different concentrations of albumin. HSA (1, 5, and 10 mg/ml) was applied at 1-hr intervals during ocular surface desiccation. After 12 hr, quantitative analysis of the fluorescein-stained area of the corneal epithelium was performed on each eye. [0 hr and 12 hr means 0 hr after scraping and 12 hr after scraping ($n = 1$) with no treatment in a desiccation room, respectively.] The scraped area at 0 hr is fixed (0.4 mm^2). Data of HSA or PBS eye drops represent the mean ± SD of 12 experiments. Paired Student's *t* test was used to determine the significance of differences. **Indicates a significant difference from the result in PBS treatment, $p < 0.01$.

region in cornea epithelial of rats caused by placement in a desiccation room was involved in apoptosis demonstrated by Hoechst 33342 staining.[15] It is also known that CCL does not survive when serum is removed from the medium. This cell death was apoptosis accompanied by caspases activation (Fig. 3). Figure 3 shows that HSA dose-dependently suppressed apoptosis.

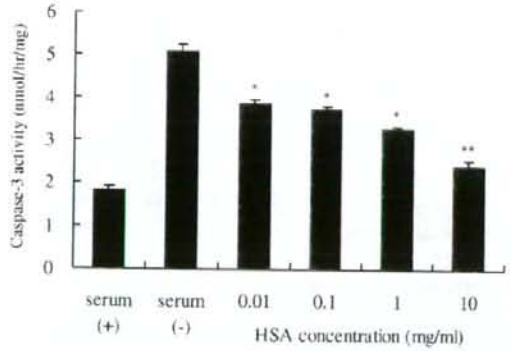

FIGURE 3 Suppression of caspase-3 activation induced by serum deprivation by HSA in CCL. Activity of caspase-3 was estimated by measuring the 7-amino-4-trifluoromethyl coumarin (AFC) production. Results are the mean ± SD ($n = 4$). Dunnett's test was used to determine the significance of differences. *Indicates a significant difference from the result in serum (−), which is incubated in serum-free Medium 199 without HSA. $p < 0.05$. **Indicates a significant difference from the result in serum (−), $p < 0.01$.

Furthermore, suppression effect of HSA on apoptosis induced by apoptosis inducers in corneal epithelial cells was investigated. It was demonstrated in many studies that the activation of caspase correlated with DNA fragmentation. DNA fragmentation of CEPI was dose-dependently induced by anti-Fas antibody (Fig. 4A) or etoposide (Fig. 4B), and this fragmentation was suppressed by 0.5 mg/ml albumin.

DISCUSSION

In this and previous studies from our group,[14] we have shown that HSA has potential for clinical treatment of dry eye patients. Because autologous serum is a good substitute for tears, we hypothesized that common components between serum and tears may be effective for treatment of dry eye. Serum albumin, which is the principal protein component of serum, is also contained in tears, which is believed to be derived from serum.[21]

The serum albumin is a multifunctional carrier protein. A number of albumin binding proteins have been identified from endothelium in various tissues.[22,23] One of the albumin binding proteins, gp60, is thought to mediate the transcytosis of albumin,[22] and this protein is shown to be distributed in alveolar epithelium in rat lung.[24] We think that gp60 is also distributed in corneal epithelium to uptake albumin in tears.

The data from rats with intact epithelium under desiccated condition was reported in our previous paper.[15] In that paper, the corneal epithelial disorder superficial punctate keratopathy and erosion were observed in most of the eye under desiccated condition for at least 5 days. However, variations in the frequency of appearance, degree of disorder, and time of appearance were noteworthy in this condition. Thus, to enable us to assess accurately the effect of pharmacological treatments, we attempted to induce uniform corneal epithelial disorder by scraping a small area of the corneal epithelium as a trigger.

Although we did not carry out the measurement of DNA fragmentation and caspase activity in the same cell lines in this study, we already reported in previous studies, in which DNA fragmentation and caspases-3 activity similarly increase in CCL induced by serum deprivation,[17] and in CEPI by etoposide or anti-Fas treatment.

Figure 2 shows 5 mg/ml of albumin is more effective than other concentrations. This result surprised us, because we expected that high concentration of albumin

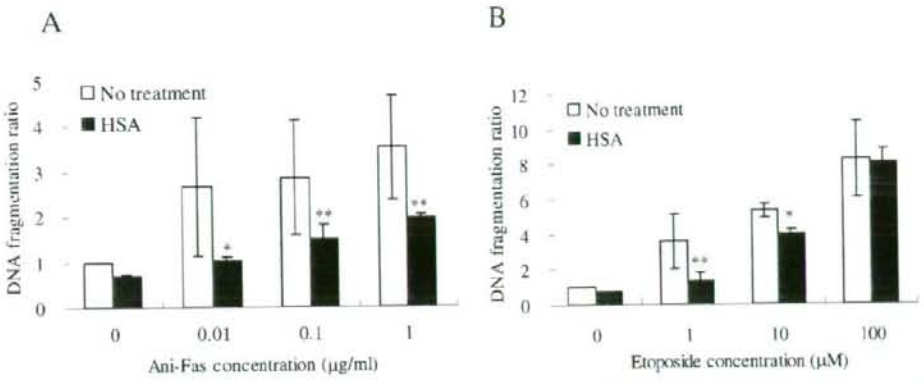

FIGURE 4 Suppression by HSA of apoptosis induced by apoptosis inducer in CEPI. CEPIs were treated with apoptosis inducers in the presence (closed bar) or absence (open bar) of 5 mg/ml HSA. DNA fragmentation, which is an index of apoptosis, was induced by apoptosis inducer, and this fragmentation was suppressed by HSA. (A) Apoptosis was induced by anti-Fas antibody; (B) induced by etoposide. Results are the mean ± SD ($n = 4$). Paired Student's *t* test was used to determine the significance of differences. *Indicates a significant difference from the result in absence of HSA (no treatment), $p < 0.05$. **Indicates a significant difference for viability from the result in absence of HSA, $p < 0.01$.

was more effective. Growth factors induce growth of cells, otherwise, high concentration of growth factors often suppress cell growth. Furthermore, it was reported that albumin containing fatty acid induced cell death.[25] We think that high concentration of albumin arrests growth of cell or induces cell death. The other reason is the albumin used in this study was purified from human serum. High concentration of HSA and/or contaminated proteins from human serum might act as an antigen in ocular surface of rat, which interferes in recovery of the ocular surface.

It is well-known that withdrawing serum from a medium causes apoptosis in cultured cells, though the precise mechanism is unclear.[26] Anti-Fas antibody induces apoptosis in Fas-expressing cells, and etoposide, which is a topoisomerase II inhibitor, induces apoptosis. These events leading to apoptosis are accompanied by caspase cascade activation.[27] HSA suppressed apoptosis induced by serum deprivation (Fig. 3) or addition of inducers (Fig. 4) in CCL or CEPI. This suppression is not shown in 0.2 mg/ml fibrinogen and 0.02 mg/ml antithrombin (data not shown). In previous studies, bovine serum albumin (BSA) survived from cell death in macrophage or renal tubular cells cultured in survival factors-free medium.[28,29] HSA protected radiation-induced apoptosis accompanied with activation of Akt signaling pathway.[30] Akt has been implicated as a major modulator of cell survival, via the phosphorylation and inactivation of proapoptotic proteins, including the BCL-2 family member BAD12 and Forkhead family transcription factors. The apoptosis suppression effect of albumin may have decreased the area of epithelial defection in the dry eye rats.

Human serum contains many different antioxidants (i.e., ascorbic acid, α-tocopherol, β-carotene, and bilirubin) that may play important roles for general health maintenance. Albumin has also been shown to possess antioxidant activity.[31,32] It was shown that 0.05 and 0.2 mg/ml BSA absorbed the peroxyl radical produced by peroxyl radical generator.[32] This concentration of BSA is comparable with that of HSA suppressing apoptosis in this study (Figs. 3 and 4). Furthermore, BSA was shown to inhibit the accumulation of reactive oxygen species after withdrawal of survival factors.[29] Other functions of albumin were shown, such as esterase-like activity[33] and enolase-like activity.[34] These antioxidant and enzyme activities may be involved with the suppression effect of HSA on apoptosis. Further studies will be required to clear the mechanism of dry eye treatment of HSA.

The precise mechanism of albumin in treatment of dry eye remains unclear. In this study, we present that HSA is useful for clinical treatment of dry eye.

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