Screening System of Blocking Agents of the Receptor for Advanced Glycation Endproducts in Cells Using Fluorescence

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Activation of the receptor for advanced glycation endproducts (RAGE) triggers cellular responses implicated in the pathogenesis of diabetic complications; blockade of RAGE has been shown to inhibit the development of diabetic complications. To develop a screening system to identify novel disruptors of advanced glycation endproducts (AGE)-RAGE binding, we used an AGE-RAGE binding system in RAGE-overexpressing cells; test compounds were screened using this system. To construct human RAGE-overexpressing cells, mouse mesangial cells (MMCs) were stably transfected with the pcDNA-human RAGE (hRAGE) vector and selected under 1 mg/mL gentamicin (G418). RAGE expression in hRAGE-overexpressing MMCs was analyzed by Western blotting with specific RAGE antibody. To identify novel disruptors of AGE-RAGE binding, 50 single compounds and AGE-bovine serum albumin (BSA)-Alexa 488 (AGE-BSA labeled with Alexa 488) were treated to the hRAGE-overexpressing MMCs. Nonbinding AGE-BSA-Alexa 488 was washed and fluorescence measured by microtiter plate reader (excitation wavelength, 485 nm; emission wavelength, 528 nm). In hRAGE-overexpressing cells, only treatment with AGE-BSA-Alexa 488 significantly increased fluorescence intensity in a dose-dependent manner. Of 50 compounds tested, genistein disrupted AGE-RAGE binding in a dose-dependent manner. This AGE-RAGE binding system using AGE-BSA-Alexa 488 in hRAGE-overexpressing cells was suitable for screening of agents that disrupt AGE-hRAGE binding.

Key words advanced glycation endproduct; receptor for advanced glycation endproduct; screening

Chronic hyperglycemia causes increased formation of advanced glycation endproducts (AGEs) in tissues of individuals with diabetes. The formation and accumulation of AGEs can accelerate the development of diabetic vascular complications.1) Approaches for the development of drugs either to prevent or treat diabetic complications have focused on inhibition of AGE formation, suppression of AGE receptor (RAGE) expression or its downstream pathways, and blockade of the AGE-RAGE interaction.2–4) Inhibitors of AGE formation such as aminoguanidine (Pimagedine) and pyridoxamine (Pyridorin) prevent diabetic nephropathy by inhibiting albuminuria, glomerular hypertrophy, and mesangial expansion in patients with type 1 diabetes and in animal models.2,5,6) The AGE-protein crosslinker breaker ALT-711 (Alagebrum) improves endothelial dysfunction in patients with isolated systolic hypertension and decreases left ventricular mass in patients with diastolic heart failure.7–9) In our previous study, 700 plant extracts and phytochemicals were tested for inhibition of AGE formation by incubating glucose and bovine serum albumin (BSA) for 14 d; several extracts were shown to prevent diabetic complications, specifically nephropathy, cataract formation, and retinopathy in experimental animal models.10–12)

RAGE is a member of the immunoglobulin superfamily that consists of three extracellular immunoglobulin-like domains (i.e., C1, C2, and C), a transmembrane helical domain, and a short intracellular, negatively charged C-terminal tail that is indispensable for RAGE signaling.13,14) The soluble form of RAGE (sRAGE) indirectly blocks AGE-RAGE binding.14) RAGE ligands such as AGE-BSA and S100b bind to RAGE with high affinity in cells and activate pro-inflammatory genes.15,16) Administration of recombinant sRAGE, consisting of the extracellular ligand-binding domain, suppresses the development of atherosclerosis in diabetic apoE-null mice and inhibits diabetic leukostasis and blood-retinal barrier breakdown.17,18) Until recently, screening for disruption of AGE-RAGE binding in cells had not been performed. The aim of this study was to develop a screening system to identify disruptors of AGE-RAGE binding in live cells using fluorescence imaging.

MATERIALS AND METHODS

Cell Culture Mouse mesangial cells (MMCs; SV40 MES13) were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM): F-12 (3 : 1) supplemented with 5% FBS (Gibco BRL, Rockville, MD, U.S.A.), 14 mM N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES), 100 units/mL penicillin, and 100 mg/mL streptomycin in a 5% CO2 humidified atmosphere at 37°C.

Establishment of Human RAGE-Overexpressing Cell Line Full-length human RAGE (NCBI GenBank No., AB036432) was amplified using sense primer 5’-TATGAA TTC ATG GCA GCC GGA ACA GCA G-3’ and antisense primer 5’-GGC CTC GAG TCA AAG CCC GCC AGT ACT AC-3’. The amplified 1.2-kb DNA fragment was digested with EcoRI and Xhol and inserted into mammalian expression vector pcDNA3.1(+)(Invitrogen, Carlsbad, CA, U.S.A.) that had been digested with the same restriction enzymes. The correct sequence and orientation of the constructs were confirmed by DNA sequencing (results not shown). Cells were transfected with the resultant pcDNA3.1(+)/human RAGE (hRAGE) plasmid using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. G418 (Gibco BRL)

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was added to the medium 48 h after transfection to select stably transfected clones. For additional experiments, the stable transfectants were cultured in media containing the same concentration of G418. Cells were collected after incubation for 1, 2, 3, 4, and 5 passages. RAGE protein from each passage was extracted; protein levels were determined by Western blot analysis using the anti-RAGE antibody (Millipore, Temecula, CA, U.S.A.).

**Immunofluorescence Staining** To determine overexpression levels of hRAGE in MMCs, either pcDNA- or pcDNA/hRAGE-transfected MMCs were fixed in 2% paraformaldehyde. Cells were permeabilized and blocked with 0.1% Triton X-100/PBS containing 2% BSA and 0.5% normal serum to reduce nonspecific adherence of antibodies. Cells were incubated in primary anti-RAGE at a dilution of 1:1000 (Millipore) in a humidified chamber at 37°C for 3 h. After incubation with primary antibody, the cells were rinsed and incubated with anti-rabbit immunoglobulin G-Texas Red (Santa Cruz, CA, U.S.A.) for protein production. Cells were resuspended in lysis buffer (pH 8.0) containing 50 mM Tris–HCl (pH 7.5), 50 mM KCl, 1 mM phenylmethylsulfonylfluoride (PMSF), 5 mM dithiothreitol (DTT), 20% (v/v) glycerol, and Triton X-100 and then sonicated. Cellular debris was removed by centrifugation. The supernatant was mixed with Ni-NTA resin (Qiagen, Japan) at its N-terminal end, for bacterial expression. The pET28a plasmid containing hRAGE DNA was then transfected into BL21 (DE3) Escherichia coli strain (Stratagene, La Jolla, CA, U.S.A.) for protein production. Cells were resuspended in lysis buffer (pH 8.0) containing 50 mM Tris–HCl (pH 7.5), 50 mM KCl, 1 mM phenylmethylsulfonylfluoride (PMSF), 5 mM dithiothreitol (DTT), 20% (v/v) glycerol, and Triton X-100 and then sonicated. Cellular debris was removed by centrifugation. The supernatant was mixed with Ni-NTA resin (Qiagen, Japan) at 4°C overnight. Binding between the hRAGE protein and its ligand and β-actin was used as internal control. (A) Western blot of hRAGE in clones 1–5 in MMCs. (B) Western blot of hRAGE in cells at passages 1–5 in hRAGE-overexpressing MMCs. (C) Immunofluorescence of hRAGE in hRAGE-overexpressing MMCs. Cell nuclei were counterstained with DAPI.

**Statistical Analysis** All data are expressed as mean±S.D. Differences between groups were examined for statistical significance using one-way analysis of variance (ANOVA) followed by Tukey post hoc test. A p-value <0.05 was considered statistically significant.

**RESULTS**

Establishment of hRAGE-Overexpressing Cell Line It was previously shown that S100b interacts with RAGE and the interaction of RAGE and its ligands increases expression of RAGE. We first established an hRAGE-overexpressing cell line using MMCs. As shown in Figs. 1A and B, hRAGE protein levels were elevated in clones 1 and 4 until passage 5. hRAGE levels in MMCs were also analyzed by immunofluorescence using an hRAGE-specific antibody (Fig. 1C).
Non-transfected and pcDNA-transfected cells (Fig. 1C) had no detectable immunofluorescence using this antibody.

AGE-BSA and hRAGE Binding in Vitro To test whether AGE-BSA could bind hRAGE, hRAGE protein was purified from E. coli and incubated with AGE-BSA in vitro. Complexes of AGE-BSA and purified hRAGE protein were tagged with anti-6x His tag antibody and confirmed by Western blotting with 6x His antibody (Fig. 2A). As shown in Fig. 2B, after pull-down of putative AGE-BSA/hRAGE complexes with anti-6x His tag antibody, Western blotting with anti-RAGE or anti-AGE antibodies was performed. IP with 6x His tag antibody in either the presence (Fig. 2B, lines 2 and 3) or absence (Fig. 2B, line 1) of AGE pulled down AGE-BSA/hRAGE complexes, confirming that RAGE and AGE do interact physically. The presence of AGE-BSA in AGE-BSA/hRAGE complexes was detected by anti-AGE antibody (Fig. 2B).

Detection of Live Cell-Based AGE-BSA/hRAGE Binding Fluorescence of hRAGE-overexpressing cells was increased after treatment with Alexa 488-labeled AGE-BSA in a dose-dependent manner. Fluorescence images, shown in Fig. 3B, show Alexa 488-labeled AGE-BSA binding to hRAGE on the cell surface. Alexa 488-labeled AGE-BSA treated with pcDNA-transfected (Fig. 3B) or untransfected cells was not detected by fluorescence. Next, to test their competition for binding to hRAGE, cells were treated with 5 µg/mL Alexa 488-labeled AGE-BSA and different doses of AGE-BSA (1, 2.5, 5, 7.5, 10 µg/mL). As the AGE-BSA concentration increased, Alexa 488-labeled AGE-BSA binding to hRAGE decreased (Fig. 3C). Fifty compounds isolated from natural products were then tested. Cellular fluorescence intensity after genistein treatment decreased in a dose-dependent manner (Fig. 3D).
DISCUSSION

There have been a number of different approaches to attenuate or prevent the biological effects of AGEs in vivo. Drugs have been developed to reduce the formation of AGEs, enhance AGE degradation, disrupt AGE cross-linking, and inhibit binding of AGE and RAGE. At present, there have been no screening systems using live cell-based AGE-RAGE binding. In the present study, we developed a screening system using a cell surface-based, Alexa 488-labeled AGE-BSA/human RAGE assay.

Previous methods used to identify drugs that block AGE formation have involved fluorescence spectroscopy. In brief, BSA and glucose are incubated with candidate compounds in phosphate buffer for 14 d. Candidate compounds that can inhibit formation of AGEs reduce fluorescence. Our previous studies identified several single compounds and extracts from natural products that inhibited AGE formation.18,20–22 To identify agents that disrupt AGE-induced protein crosslinking in vitro, candidate compounds were added to a collagen-coated surface that had been pretreated with AGE-BSA. AGE-collagen cross linking was detected using specific anti-AGE and horseradish peroxidase-linked antibodies. The AGE cross-linking disruptor N-phenacylhydrazolium bromide (PTB) reacts with and cleaves covalent AGE-derived protein crosslinks.23 Most of these methods have been performed in vitro. However, our screening system examined live hRAGE-overexpressing cells using fluorescence imaging.

In this study, we established a full-length hRAGE-overexpressing cell line (Fig. 1) and tested the binding competition between AGEs and Alexa-labeled AGE-BSA to hRAGE (Fig. 3C). Increased concentrations of AGE-BSA reduced the binding of Alexa-labeled AGE-BSA to hRAGE in live cells. Single compounds were then added to the cells, and one [genistein (4',5',7'-trihydroxyisoflavone, a strong antioxidant isoflavinooid found in soy beans and the roots of Pueraria lobata)] significantly disrupted AGE/hRAGE binding (Fig. 3D). In this study, we show that genistein disrupts AGE-RAGE binding. Genistein exerts inhibitory effects on AGE formation and aldose reductase activity in vitro.11,20 Further studies, such as in vivo studies in diabetic animal models and toxicological investigations, are required additionally to elucidate its mechanism of action and effect.

Researchers have shown 20–30% higher AGE levels in individuals with uncomplicated diabetes and 40–100% higher AGE levels in subjects with complicated diabetes.23,24 AGE enhances reactive oxygen species (ROS) generation and promotes higher levels of AGE via the NADPH oxidase pathway. AGE-RAGE binding stimulates the generation of ROS and inflammatory mechanisms that enhance AGE formation. AGE-RAGE interactions play important roles in the development and progression of diabetic complications. Prevention of AGE-RAGE-dependent signaling may hold the key to tissue damage often seen in diabetic disorders. Thus our novel system is a promising tool to screen drugs designed to disrupt signaling pathways that lead to diabetic complications.

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