Inhibition of Diabetic Leukostasis and Blood–Retinal Barrier Breakdown with a Soluble Form of a Receptor for Advanced Glycation End Products

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PURPOSE. The interaction of advanced glycation end products (AGEs) with their receptors is hypothesized to be involved in the development of diabetic retinopathy. In the present study, the role of an AGE receptor, RAGE, was investigated in the development of diabetic retinopathy in vivo.

METHODS. C57/Bj6 and RAGE-transgenic mice that carried human RAGE genetic DNA under the control of the murine flk-1 promoter were made diabetic with streptozocin. Three months after the onset of diabetes, the soluble form of RAGE (sRAGE) or mouse serum albumin was injected intraperitoneally at 100 μg/d for 14 consecutive days. After the final injection, blood–retinal barrier breakdown, retinal leukostasis, expression of VEGF and ICAM-1, and expression of RAGE in the retina were investigated.

RESULTS. Blood–retinal barrier breakdown and increased leukostasis were associated with the experimental diabetes in the C57/Bj6 mice. These changes were significantly augmented in RAGE-transgenic mice. The blood–retinal barrier breakdown and leukostasis in the diabetic C57/Bj6 and RAGE-transgenic mice were accompanied by increased expression of VEGF and ICAM-1 in the retina. The systemic administration of sRAGE significantly inhibited blood–retinal barrier breakdown, leukostasis, and expression of ICAM-1 in the retina in both the diabetic C57/Bj6 and RAGE-transgenic mice. The expression of RAGE was slightly increased in the retinal vessels in diabetic or RAGE-transgenic mice. Furthermore, a strong induction of RAGE was observed in the retinal vessels of diabetic RAGE-transgenic mice.

CONCLUSIONS. This study further demonstrates the role of the AGES and RAGE axis in blood–retinal barrier breakdown and the retinal leukostasis, which are characteristic clinical symptoms of diabetic retinopathy. Furthermore, these data demonstrate that blocking AGES bioactivity may be effective for the treatment of diabetic retinopathy. (Invest Ophthalmol Vis Sci. 2007;48:858 – 865) DOI:10.1167/iovs.06-0495

Diabetic retinopathy is a leading cause of blindness and visual impairment in the developed world and its incidence is increasing. Advances have been made in the treatment of diabetic retinopathy; however, the number of visually impaired and blind patients with the disease continues to increase. Therefore, it is important to understand the pathogenic mechanisms underlying diabetic retinopathy so that novel, more effective therapeutic and preventative strategies can be developed.

Diabetes induces various complications, including nephropathy, neuropathy, retinopathy, and microangiopathy. The clinical complications vary considerably; however, they may share common underlying mechanisms. Potential biochemical pathways include enhanced polyol metabolism, activation of PKC-β, increased oxidative stress, and the accumulation of advanced glycation end products (AGEs). A combination of these mechanisms may underlie the pathogenesis of diabetic retinopathy. The inhibition of these pathways by aldose reductase inhibitors, PKC-β inhibitors, antioxidants, and/or amnoguanidine, an inhibitor of AGES generation, has been shown to ameliorate diabetic retinopathy in experimental models.

AGEs may play an important role in the development of diabetic complications and the aging process. AGES are the final product of the nonenzymatic reaction of proteins and reducing sugars. With diabetes and age, AGES tend to accumulate in the body. Local accumulation of AGES is speculated to have a central role in the development of diabetic nephropathy and microangiopathy. In addition, accumulation of AGES is reported in degenerative disorders such as Alzheimer’s disease. Similarly, the accumulation of AGES has been proposed to have an important role in the development of diabetic retinopathy. Stitt et al. showed the accumulation of AGES in retinal vessels by using an antibody specific to AGES. In addition, blood–retinal barrier breakdown, a characteristic sign of diabetic retinopathy, was seen in nondiabetic animals that received intravenous injections of AGES. These data suggest that the formation and deposition of AGES in the retina lead to some aspects of diabetic retinopathy.

The biological effects of AGES are in part mediated by the specific cell surface receptors. Several AGES receptors are known such as the receptor for AGE (RAGE), galectin-3, CD36, and the macrophage scavenger receptor. In addition, it has been suggested that the interaction of AGES with their receptors is involved in the pathogenesis of diabetic complications. The interaction of RAGE with AGES has been shown to induce changes in retinal histology and electroretinogram in experimental diabetic animals. Further studies are needed to demonstrate the direct involvement of RAGE in diabetic retinopathy.
The purpose of this study was to analyze the effect of RAGE, a major receptor for AGEs, in the development of diabetic retinopathy in vivo. To augment the interaction of AGEs and RAGE, RAGE-transgenic mice with increased levels of RAGE expression in systemic blood vessels were analyzed. In addition, in another group of experimental animals, the interaction of AGEs with RAGE was accomplished through the use of a soluble form of RAGE (sRAGE). The diabetic retinopathy endpoints studied included blood-retinal barrier breakdown and the adhesion of leukocytes to the retinal vascular wall (retinal leukostasis).

**MATERIALS AND METHODS**

**Animals**
Male C57/Bl6 and RAGE-transgenic mice of 6 weeks of age with the genetic background of C57/Bl6 were used. Mice were obtained from The Jackson Laboratories (Bar Harbor, ME) and the University of Kanazawa (Kanazawa, Japan), respectively, and housed in standard non-pathogen-free conditions. The RAGE-transgenic mice had been generated as previously described. Briefly, these mice carry human RAGE genetic DNA under the control of the murine Ikb-1 promoter. The expression of RAGE is increased in the systemic vasculature, including the retina. All experimental protocols were approved by the Animal Care and Use Committee of the Massachusetts Eye and Ear Infirmary and were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Experimental Diabetes**
Experimental diabetes was induced as previously described. Briefly, intraperitoneal injections of 55 mg/kg of streptozocin (Sigma-Aldrich, St. Louis, MO) diluted in sterile citrate buffer (0.05 M, pH 4.5) were administered daily for 4 consecutive days. Control mice were injected with citrate buffer alone. At 7 days and 1, 2, and 3 months after the first injection of streptozocin or citrate buffer, nonfasting whole blood was removed from the tail vein. Animals with blood glucose levels more than 300 mg/dL at every point were deemed diabetic.

The body weight and blood glucose levels in control, diabetic nontransgenic, normoglycemic RAGE-transgenic, and diabetic RAGE-transgenic mice were 34 ± 3.8 g/165 ± 14.9 mg/dL, 35 ± 4.1 g/493 ± 38.9 mg/dL, 35 ± 2.7 g/155 ± 12.2 mg/dL, and 36.4 ± 4.5 g/511 ± 28.8 mg/dL, respectively. There was no significant difference in the body weight of the animals in the four groups (Mann-Whitney test with the Dunn procedure as a multiple-comparison test.). In addition, there was no significant difference in the blood glucose level of diabetic nontransgenic and diabetic RAGE-transgenic mice (two-way factorial ANOVA).

**Soluble RAGE**
Soluble RAGE was prepared by using a baculovirus expression system using S9 insect cells (BD-Clontech, Palo Alto, CA), as previously described. Purified murine sRAGE was dialyzed against phosphate-buffered saline (PBS) and sterile-filtered (0.22 μm). Previous work has shown that sRAGE prepared as described herein will block the binding of AGEs to its receptors. Three months after induction of diabetes, the experimental mice received intraperitoneal injections of 100 μg sRAGE daily for 14 consecutive days. Mouse serum albumin (Sigma-Aldrich) was used as a control at equimolar concentrations. Twenty-four hours after the final injection of sRAGE, the following experiments were performed on all experimental groups.

**Quantification of Retinal Leukostasis**
Deep anesthesia was introduced with 50 mg/kg of pentobarbital sodium. The chest cavity was carefully opened and the left ventricle was entered with a 20-gauge perfusion cannula fixed with a clamp. The right atrium was opened with an 18-gauge needle to achieve outflow. First, 250 mL/kg of PBS was perfused to remove the nonadherent leukocytes in the retinal vasculature. Fluorescein isothiocyanate-coupled concanavalin A lectin (20 μg/mL in PBS, total concentration 5 mg/kg body weight; Vector Laboratories, Burlingame, CA) was perfused to label the adherent leukocytes in the retinal vasculature. Finally, 250 mL/kg of PBS was perfused to wash out the excess concanavalin A. The retinas were carefully flatmounted in a water-based fluorescent antifade medium (Southern Biotechnology, Birmingham, AL) and imaged via fluorescence microscopy (fluorescein isothiocyanate filter, Axiosvert; Carl Zeiss Meditec, Inc., Dublin, CA). The total number of adherent leukocytes in the retinal vasculature was counted. Counting the number of static leukocytes in the retina was performed in a double-blind manner.

**Blood–Retinal Barrier Breakdown**
Blood-retinal barrier breakdown was evaluated as previously described. Briefly, 45 mg/mL Evans blue (Sigma-Aldrich) was injected through the tail vein. Two hours after the injection, the chest cavity was carefully opened, and 0.2 mL of total blood was obtained from the left ventricle. Then the animals were perfused with PBS for 2 minutes to wash out the Evans blue in the vessels completely. The retinas were then carefully isolated under the dissecting microscope without contaminating the retinas with aqueous humor. Blood-retinal barrier breakdown was calculated using the concentration of Evans blue in the blood and the retina.

**Expression of VEGF and ICAM-1**
Retinal VEGF and intercellular adhesion molecule (ICAM)-1 levels were measured using an enzyme-linked immunosorbent assay. Retinal lysates were prepared by washing the tissues with PBS three times. The retinas were homogenized in 100 μL of solution consisting of 20 mM imidazole hydrochloride, 100 mM KCl, 1 mM MgCl₂, and 1 mM EGTA. The solution was supplemented with a cocktail of protease inhibitors (Complete; Roche, Basel, Switzerland) before use. The lysate was cleaned of debris by centrifugation at 14,000 g for 30 minutes at 4°C, and the supernatant was assayed for VEGF and ICAM-1. The concentration of total protein in the samples was determined by the bovine serum albumin (BSA) assay (Micro BCA; Pierce, Rockford, IL). Supernatant VEGF levels were determined by using a sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN).

The level of ICAM-1 in the lysate was also determined by ELISA. Flat-bottomed 96-well microtiter plates (Immunoplate 1 96-F; Nunc, Naperville, IL) were coated with 50 μL/well (1 ng/mL) of the specific rabbit anti-ICAM-1 antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) in a coating buffer containing 600 mM NaCl, 260 mM H₃PO₄, and 0.08 N NaOH (pH 9.6) for 16 to 24 hours at 4°C. Nonspecific sites were then blocked with 2% BSA in PBS for 1 hour at 37°C. Fifty-microliter retinal samples prepared as previously described were added and incubated for 1 hour at 37°C. After washing with PBS, 50 μL of biotinylated anti-ICAM-1 was added, and the samples were incubated for 1 hour at 37°C. The plates were washed with PBS and streptavidin-peroxidase conjugate (1/1000; R&D Systems) was added. Tetramethylbenzidine (100 μL; Biosource, Nivelles, Belgium) was incubated for 10 minutes at room temperature before 100 μL of blocking solution (Biosource) was added. Fixed optical density was read at 450 nm with a spectrophotometer (model Lambda Bio 40; Perkin Elmer, Boston, MA).

**Serum Concentration of AGE**
The concentration of N²-carboxymethyllysine (CML) in the hemoglobin and serum was determined with a competitive ELISA as previously described. One unit per microliter of CML corresponded to a protein...
concentration of 1 μg/mL CML-BSA. Standard AGE-BSA was made as previously described. Bovine serum albumin (fraction V, fatty-acid free, low endotoxin BSA; Roche GmbH, Mannheim, Germany) was incubated with 50 mM glucose in PBS for 6 weeks at 37°C in sterile condition. At the end of incubation, the solution was dialyzed against PBS, sterile-filtered through 0.22-μm nylon filters (Pall Corp., Ann Arbor, MI), aliquoted, and stored at −70°C until used. Specific monoclonal antibody to CML (clone 6D12) was purchased from Wako Pure Chemical (Tokyo, Japan).

Immunohistochemical Localization of RAGE

Immunohistochemical localization of RAGE was investigated in RAGE-transgenic and nontransgenic mice, with or without induced diabetes. Multiple 3-μm-thick sections of 10% formalin-fixed, paraffin-embedded specimens were prepared. The immunohistochemical localization of RAGE was examined using the peroxidase-labeled polymer for the goat antibody (Mouse Max-PO(G) Kit; Nichirei Co. Ltd., Tokyo, Japan). The goat polyclonal antibody recognizing the V domain of RAGE was purchased (dilution 1:800; Chemicon, Temecula, CA). Immunohistochemical procedures were based on techniques described previously. Negative controls were prepared by replacing polyclonal antibodies to CML (clone 6D12) was purchased from Wako Pure Chemical (Tokyo, Japan).

Statistical Analyses

All results were expressed as the mean ± SD. The data were processed for statistical analysis with the Mann-Whitney test with the Dunn procedure as a multiple-comparison test. Differences were considered statistically significant at *P < 0.05.

RESULTS

Blood–Retinal Barrier Breakdown

Figure 1 shows blood-retinal barrier function as evaluated by Evans blue assay. In nontransgenic mice with 3 months of diabetes, the degree of the blood-retinal barrier breakdown was 2.5-fold greater than that in the nontransgenic normoglycemic mice. Although the RAGE-transgenic mice were normoglycemic, the degree of the blood-retinal barrier breakdown was more than that in the nontransgenic hyperglycemic mice. In the RAGE-transgenic mice, hyperglycemia increased the degree of blood-retinal barrier breakdown to approximately 2.5 times higher than that of RAGE-transgenic normoglycemic mice. In addition, sRAGE significantly reduced the degree of the blood-retinal barrier breakdown in diabetic nontransgenic and diabetic RAGE-transgenic mice.

Leukostasis

In diabetes, ICAM-1 expression increases in the retinal vasculature and leukostasis increases. Figure 2A is representative of concanavalin-A lectin staining in the retinal vasculature in nontransgenic diabetic mice. The adherent leukocytes were detected on arterioles, venules, and capillaries. Figure 2B shows the total number of adherent leukocytes per retina in the experimental model. The number of adherent leukocytes increased threefold in the diabetic nontransgenic mice compared with the control nondiabetic mice. Even under normoglycemic conditions, the number of adherent leukocytes in the retina in the RAGE-transgenic mice was more than that in the nontransgenic diabetic mice. The number of adherent leukocytes increased twofold in the diabetic RAGE-transgenic mice compared with the nondiabetic ones. Systemic application of sRAGE significantly reduced the number of adherent leukocytes in the retinas of the diabetic nontransgenic and diabetic RAGE-transgenic mice.

VEGF and ICAM-1 Expression

Figures 3A and 3B show the retinal protein levels for VEGF and ICAM-1, respectively, in retinas evaluated with enzyme-linked immunoassay. Figure 3 shows the total number of adherent leukocytes per retina in the experimental model. The VEGF and ICAM-1 levels increased approximately 2- and 1.5-fold, respectively, in the diabetic nontransgenic mice compared with the control. Even when normoglycemic, the retinal VEGF
and ICAM-1 levels were greater than those of the nontransgenic diabetic mice. The VEGF and ICAM-1 levels increased 1.5- and 1.8-fold, respectively, in diabetic RAGE-transgenic mice compared with nondiabetic ones. Systemic treatment with sRAGE decreased the retinal VEGF level in diabetic nontransgenic and diabetic RAGE-transgenic mice; however, the difference was not statistically significant. In addition, systemic treatment with sRAGE significantly reduced the retinal ICAM-1 level in the diabetic nontransgenic and diabetic RAGE-transgenic mice.

**Concentration of Serum AGE**

Figure 4 shows the concentration of CML, one of the major components of AGEs in serum. Diabetic mice had a tendency toward an increased concentration of AGEs in serum compared with normoglycemic mice; however, the changes were not statistically significant.

**Immunohistochemical Localization of RAGE**

Figure 5 shows the immunohistochemical localization of RAGE in RAGE-transgenic and nontransgenic mice with or without induced diabetes. In normoglycemic nontransgenic mice, the expression of RAGE was detected predominantly in the inner and outer plexiform layers (Fig. 5A). The expression level of RAGE in retinal vessels was almost the same as that of the inner and outer plexiform layers (Fig. 5A). In diabetic nontransgenic mice, the expression level of RAGE in the inner and outer plexiform layers was almost the same as that of normoglycemic mice.
nontransgenic mice (Fig. 5B). However, the expression level of RAGE was slightly increased in the vessels in the outer plexiform layer (Fig. 5B). In normoglycemic RAGE-transgenic mice, the expression level of RAGE was slightly increased in retinal vessels in the ganglion cell and outer plexiform layers (Fig. 5C). In diabetic RAGE-transgenic mice, strong induction of the expression of RAGE was detected in retinal vessels in the ganglion cell and the outer plexiform layers (Fig. 5D). In negative control specimens, immunodetection with nonimmunized IgG instead of a specific antibody to RAGE showed no reaction in the retina of any animals (data not shown).

**DISCUSSION**

In this study, the direct involvement of RAGE in the development of diabetic retinopathy was examined. As no specific inhibitors of RAGE have been found, the biological effect of RAGE was analyzed by using two methods. First, quantifiable diabetic retinopathy endpoints were examined in wild-type mice and RAGE-transgenic mice. The expression of RAGE in the systemic vasculature was several times higher than in the wild-type mice. In a second set of experiments, the extracellular domain of RAGE (sRAGE) was administered systemically.
IL-1, and TGF-β induce the expression of various cytokines including VEGF, which is important in the development of diabetic microangiopathy.31 In addition, AGE-modified proteins can induce cytotoxicity by binding cell surface receptors.6,19–21,24,27 RAGE, galectin-3, macrophage scavenger receptor, and CD36 have been reported as specific receptors for AGEs. Among them, the interaction of AGEs and RAGE has been reported to play an important role in the development of diabetic microangiopathy.6,25 RAGE was purified and cloned as a member of the immunoglobulin superfamily recognizing AGE-modified protein.6,18 The intracellular signal transduction of RAGE has still not been fully determined; however, the interaction of AGEs and RAGE leads to the increased oxidative stress and the induction of apoptosis.30 Subclinical retinal leukostasis and blood-retinal barrier breakdown are two other important diseases.

Some AGE-modified proteins are known to lose their biological activity.31 In addition, AGE-modified proteins can induce cytotoxicity by binding cell surface receptors.6,19–21,24,27 RAGE, galectin-3, macrophage scavenger receptor, and CD36 have been reported as specific receptors for AGEs. Among them, the interaction of AGEs and RAGE has been reported to play an important role in the development of diabetic microangiopathy.6,25 RAGE was purified and cloned as a member of the immunoglobulin superfamily recognizing AGE-modified protein.6,18 The intracellular signal transduction of RAGE has still not been fully determined; however, the interaction of AGEs and RAGE leads to the increased oxidative stress and the activation of NF-κB.52–55 The activation of NF-κB is known to induce the expression of various cytokines including VEGF, IL-1, and TGF-β, and various adhesion molecules including CD18, ICAM-1, and VCAM-1.32,53 These changes may well work in concert to initiate and amplify diabetic complications.

Blocking the interaction of AGEs and RAGE with sRAGE is known to ameliorate some diabetic complications in experimental models.16,24 We previously showed that sRAGE inhibits the AGE-induced activation of human umbilical vein endothelial cells (HUVECs) and the adhesion of leukocytes to the HUVECs.16 Lalla et al.24 have shown in vivo data that the systemic application of sRAGE inhibits atherosclerosis and periodontal disease in diabetic animals. In the present study, sRAGE inhibited blood-retinal barrier breakdown and leukostasis, as well as the expression of ICAM-1. This suggests that sRAGE may prove useful in the treatment of diabetic retinopathy. The data also confirm that the interaction of AGEs and RAGE is important in the development of some aspects of diabetic retinopathy.

The importance of the AGERAGE axis in the development of diabetic retinopathy was clearly shown by Barile et al.22 The results of the present study also support the hypothesis that the interaction of RAGE and AGEs is involved in the pathogenesis of diabetic retinopathy. First, the expression of RAGE was increased not only in the RAGE-transgenic mice but also in the diabetic mice. In contrast, the expression level of RAGE in the inner and outer plexiform layers was not significantly different in the animals. The fact indicates that the interaction of AGEs and RAGE increased in both RAGE-transgenic and diabetic mice even though the concentration of AGEs was not significantly different in these animals. To provide direct causal proof that RAGE is involved in diabetic retinopathy, we compared the degree of blood-retinal barrier breakdown and leukostasis in C57/BJ6 wild-type and RAGE-transgenic mice.23 In general, a low-level expression of RAGE was present on the normal vascular endothelium; however, it increased with diabetes. In RAGE-transgenic mice, the expression level of RAGE increased not only in the RAGE-transgenic mice but also in the diabetic mice. In contrast, the expression level of RAGE in the inner and outer plexiform layers was not significantly different in the animals. The fact indicates that the interaction of AGEs and RAGE increased in both RAGE-transgenic and diabetic mice even though the concentration of AGEs was not significantly different in these animals.
RAGE are sufficient to induce the blood-retinal barrier breakdown and leukostasis in these animal models.

The interaction of AGEs and RAGE is known to induce cellular change relating to diabetic complications. Activation of RAGE on the vascular endothelial cells leads to increased oxidative stress with increased production of reactive oxygen species. The increased oxidative stress induces the activation of NF-κB and thus the expression of various proteins, including VEGF, CD18, ICAM-1, and IL-1β. For this reason, the activation of RAGE is speculated to play a central role in the blood–retinal barrier breakdown and in the leukostasis seen in the present study. However, with diabetes, various factors may be involved in the increased oxidative stress and the activation of NF-κB. For example, the polylol pathway has been shown to lead to oxidative stress and the activation of NK-κB. The interaction of AGEs and RAGE may represent one of several factors that leads to diabetic complications.

In conclusion, taken together, the data indicate that interaction of AGEs with RAGE leads to leukostasis and blood–retinal barrier breakdown, characteristic findings in diabetic retinopathy. RAGE may serve as a pharmacological target for the treatment and prevention of diabetic complications in the eye.

References


