Receptor for Advanced Glycation End Products Mediates Inflammation and Enhanced Expression of Tissue Factor in Vasculature of Diabetic Apolipoprotein E–Null Mice


Abstract—Advanced glycation end products (AGEs) and their cell surface receptor, RAGE, have been implicated in the pathogenesis of diabetic complications. Here, we studied the role of RAGE and expression of its proinflammatory ligands, EN-RAGEs (S100/calgranulins), in inflammatory events mediatiing cellular activation in diabetic tissue. Apolipoprotein E–null mice were rendered diabetic with streptozotocin at 6 weeks of age. Compared with nondiabetic aortas and kidneys, diabetic aortas and kidneys displayed increased expression of RAGE, EN-RAGEs, and 2 key markers of vascular inflammation, vascular cell adhesion molecule (VCAM)-1 and tissue factor. Administration of soluble RAGE, the extracellular domain of the receptor, or vehicle to diabetic mice for 6 weeks suppressed levels of VCAM-1 and tissue factor in the aorta, in parallel with decreased expression of RAGE and EN-RAGEs. Diabetic kidney demonstrated increased numbers of EN-RAGE–expressing inflammatory cells infiltrating the glomerulus and enhanced mRNA for transforming growth factor-β, fibronectin, and α1 (IV) collagen. In mice treated with soluble RAGE, the numbers of infiltrating inflammatory cells and mRNA levels for these glomerular cytokines and components of extracellular matrix were decreased. These data suggest that activation of RAGE primes cells targeted for perturbation in diabetic tissues by the induction of proinflammatory mediators. (Arterioscler Thromb Vasc Biol. 2001;21:905-910.)

Key Words: receptor for advanced glycation end products ■ glycation ■ diabetes ■ nephropathy ■ atherosclerosis

The receptor for advanced glycation end products (RAGE), a multifunctional member of the immunoglobulin superfamily of cell surface molecules, has been implicated in the pathogenesis of diabetic complications.1–3 RAGE was first described as a central cell surface interaction site for advanced glycation end products (AGEs), the products of nonenzymatic glyoxidation of proteins/lipids that accumulate in the plasma and tissues of patients with diabetes. A view is emerging that exaggerated inflammatory responses are important in the pathogenesis of the complications of diabetes, such as in the macrovasculature,4 bacteria-infected periodontium,5 and wound tissue.6 It was in this context that we speculated that proinflammatory ligands of RAGE (EN-RAGEs) and related members of the S100/calgranulin family7 may, along with AGEs, mediate inflammatory responses that trigger cellular activation and dysfunction in diabetic tissues.

To examine the effects of RAGE and its ligands on vascular function, we studied 2 tissues especially susceptible to long-term complications in diabetes, the aorta and kidney. We have previously demonstrated that apoE-null mice displayed increased atherosclerotic lesion area and complexity at the aortic sinus on induction of diabetes.8 AGE-enriched diabetic atherosclerotic lesions were characterized by increased numbers of macrophages and vascular smooth muscle cells compared with lesions in euglycemic mice, suggesting that inflammatory responses were accelerated in diabetes. A central role for RAGE was suggested by the finding that the administration of soluble RAGE (sRAGE) to diabetic mice caused dose-dependent suppression of the lesion area and complexity, in parallel with decreased levels of plasma and tissue AGEs.8 Importantly, multiple reports have suggested that a range of AGEs, especially Nε-(carboxymethyl)lysine, accumulate in the glomerulus and interstitium of the diabetic kidney.9,10 Nε-(Carboxymethyl)lysine–modified adducts of proteins, the most prevalent AGEs found in vivo,11,12 are signal-transducing ligands of RAGE.13 Because accumulation of AGEs has been linked to sites of enhanced expression of RAGE,14 it was not surprising that although RAGE is present at low levels in normal kidney, its expression is increased in diabetic glomeruli, particularly within podocytes.15 However, the recent observation that glomerular macrophage recruit-

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ment occurs in the first days of streptozotocin diabetes in rats has suggested that inflammatory factors may trigger cellular activation in the kidney.

These considerations led us to examine the presence of proinflammatory EN-RAGEs and RAGE in macrovascular and kidney tissue in diabetic apoE-null mice and to test the effects of ligand-RAGE blockade. We speculate that accumulating RAGE ligands engaging the receptor trigger inflammatory responses, thereby stimulating cellular activation in organs primed for the development of diabetic complications.

Methods

Animal Model, Induction of Diabetes, and Blockade of RAGE

Male apoE-null mice (C57BL/6 background) were purchased from the Jackson Laboratories (Bar Harbor, Me). At 6 weeks of age, mice received streptozotocin (Sigma Chemical Co) dissolved in citrate buffer (0.2 mol/L, pH 4.5; 60 mg/kg IP) for 5 consecutive days or the same schedule of citrate buffer. On documentation of diabetes (blood glucose ~250 mg/dL), diabetic mice were treated once daily with murine sRAGE (100 μg/d) that was prepared, purified, and devoid of endotoxin, as previously described.7,8 or equal amounts of vehicle, murine serum albumin (MSA, Sigma). Treatment was continued until euthanasia, 6 weeks after documentation of diabetes (~14 weeks of age). Blood was retrieved for preparation of plasma and red blood cells. Levels of cholesterol (Boehringer-Mannheim), triglycerides (Sigma), and glycosylated hemoglobin (EG&G Wallac, Inc) were determined. Diabetic mice did not demonstrate ketonuria and gained weight during the course of the study.

Tissue Collection and Preparation

At euthanasia, the kidney and aorta were removed and stored at −80°C. Aorta was retrieved in its entirety from the proximal arch to the iliac bifurcation. Tissue was placed in buffered paraformaldehyde (4%) in preparation for immunohistochemistry, or lysates were prepared for immunoblotting by homogenizing tissue (Brinkmann) in PBS containing complete proteinase inhibitor (Boehringer-Mannheim). Protein concentrations were measured by assay (Bio-Rad).

Immunoblotting

Protein extracts were subjected to SDS-PAGE (Novex/Invitrogen) and transfer of the contents of the gels to nitrocellulose membranes (Novex/Invitrogen). Immunoblotting was performed as described.7 Sites of antibody binding were identified by using chemiluminescence (ECL, Amersham-Pharmacia). Molecular weights (approximate) of the bands were identified by simultaneous electrophoresis of molecular markers (Amersham). Bands were scanned into a laser densitometer, and quantification was performed by use of ImageQuant software (Molecular Dynamics). Antibodies used were as follows: goat anti-human vascular cell adhesion molecule (VCAM)-1 IgG (0.4 μg/mL, Santa Cruz Biotechnology), goat anti-rat tissue factor IgG (14 μg/mL, generously supplied by Drs Walter Kisiel [University of New Mexico, Albuquerque] and Vijay Rao [University of Texas Health Sciences at Tyler]), rabbit anti-murine RAGE IgG (4.1 μg/mL), and rabbit anti–EN-RAGE IgG (4 μg/mL).

Northern Blotting

Kidney tissue was homogenized in TRIZOL reagent (Life Technologies), and total RNA was isolated. The concentration and purity of the RNA were established by measuring optical absorbance at 260/280 nm (Ultra-spec Plus spectrophotometer; AmershamPharmacia). Total RNA (30 μg) was loaded per well of denaturing formaldehyde gels by use of reagents provided by Ambion. Northern blotting was performed as previously described. The cDNAs for mRNA for αI (IV) collagen, fibronectin, and transforming growth factor-β were provided by Dr F.N. Ziyadeh (University of Pennsylvania).20 To control for loading and transfer of RNA, membranes were stripped, and a radiolabeled cDNA probe for mouse β-actin was used.

Preparation of Nuclear Extract and EMSA

Nuclear proteins from aortic tissue were extracted by using an NE-PER extraction kit ( Pierce) and subjected to electrophoretic mobility shift assay (EMSA).7,13 Samples from binding reactions were loaded onto acrylamide gels (6%). Gels were dried and exposed to Kodak BioMax MS film at −80°C for 17 hours.

Immunohistochemistry

Aortas and kidneys were paraformaldehyde-fixed for 16 hours; paraffin-embedded sections (5 μm thick) were prepared and subjected to immunohistochemistry with the following antibodies: goat anti-human VCAM-1 IgG (2 μg/mL), goat anti-rat tissue factor IgG (14 μg/mL), and rabbit anti–EN-RAGE IgG (40 μg/mL). Respective controls with use of the indicated species of nonimmune IgG were performed; no specific immunostaining with control IgGs was observed (data not shown).

Statistical Analysis

Data are reported as mean ± SE. Data were analyzed by ANOVA and, as indicated, subject to post hoc comparisons by using 2-tailed t tests.

Results

Male apoE-null mice were rendered diabetic by the administration of streptozotocin. On documentation of diabetes, mice received either murine sRAGE or MSA (100 μg/d) for 6 weeks. We used 100 μg/d sRAGE because previous experiments in a murine model of delayed-type hypersensitivity suggested that maximal suppression of RAGE-mediated inflammation was observed at this dose.7 Mice were euthanized 6 weeks after initiation of treatment with sRAGE or MSA, at which time the mice were ~14 weeks of age. At euthanasia, aortic tissue in its entirety, from the proximal arch to the iliac bifurcation, and kidney tissue were retrieved for analysis of markers of inflammation and cellular activation.

Consistent with previous observations that expression of RAGE was increased in diabetic tissue, diabetic mice displayed an ~2.0-fold increase in levels of RAGE antigen (M, ~55 kDa) in the aorta by immunoblotting after 6 weeks of diabetes (Figure 1a). Immunohistochemistry localized the sites of RAGE expression to vascular smooth muscle and endothelia, as well as adherent mononuclear cells (data not shown). The promoter of the gene encoding RAGE is enriched in functional elements, such as binding sites for nuclear factor (NF)-κB.21 Previous studies in transiently transfected endothelial and smooth muscle cells bearing luciferase-reporter promoter constructs have suggested that NF-κB elements within the RAGE promoter are responsive to a range of inflammatory stimuli.21 Thus, we tested whether blockade of RAGE would suppress levels of receptor in diabetic aorta. Aortic tissue retrieved from mice treated with sRAGE demonstrated decreased levels of RAGE antigen by immunoblotting; the expression of RAGE was reduced nearly to the levels observed in nondiabetic control mice (Figure 1a).

Because previous studies have demonstrated an increased accumulation of vascular AGEs in diabetes,8 we tested the concept that induction of diabetes was associated with increased expression of proinflammatory EN-RAGEs in aortic tissue. Levels of EN-RAGE antigen (M, ~12 kDa) were enhanced ~2.5-fold in diabetic aortas compared with control
aortas (Figure 1b). That RAGE was importantly involved in diabetes-associated increases in aortic EN-RAGEs was supported by the finding that levels of EN-RAGEs were reduced in sRAGE-treated diabetic aorta (Figure 1b). Immunohistochemistry studies localized sites of EN-RAGE expression to inflammatory cells, such as mononuclear phagocytes and polymorphonuclear leukocytes, infiltrating the vascular aorta. Increased numbers of EN-RAGE–expressing inflammatory cells were adherent to the aortic endothelium in diabetic mice compared with control mice (online Figure I; please see http://atvb.ahajournals.org). In the presence of sRAGE, numbers of EN-RAGE–expressing inflammatory cells adherent to aortic endothelium were significantly decreased (Figure 1).

The attraction of increased numbers of EN-RAGE–expressing inflammatory cells to the diabetic aorta suggested that levels of proinflammatory adhesion molecules were enhanced in diabetes. Because ligation of RAGE by either AGEs or EN-RAGEs was previously linked to transcriptional/translational regulation of VCAM-1 in vitro, we assessed expression of this adhesion molecule in vivo. An ~2.6-fold increase in VCAM-1 antigen (M₆ ~95 kDa) was observed by immunoblotting in diabetic aortas compared with control aortas (Figure 2). Immunohistochemical analysis of aortic tissue with the use of anti–VCAM-1 IgG supported these findings and localized the VCAM-1 antigen to endothelia and vascular smooth muscle (online Figures III and II, respectively; please see http://atvb.ahajournals.org). A central role for RAGE in diabetes-associated upregulation of proinflammatory adhesion molecules was supported by suppression of levels of VCAM-1 in diabetic mice treated with sRAGE (Figure 2a and online Figure IV, which can be accessed at http://atvb.ahajournals.org).

Figure 1. Induction of diabetes in apoE-null mice results in enhanced expression of RAGE and EN-RAGES in the aorta: suppression by blockade of RAGE. Diabetic apoE-null mice were treated with MSA or sRAGE as described. At euthanasia, aortic tissue was retrieved, lysates were prepared, and immunoblotting was performed for RAGE (a) or EN-RAGE (b). The approximate M₆ of standard molecular weight markers is shown. Representative bands from each condition are shown; immunoblots from 3 mice per condition were performed. Densitometric analysis is indicated, and mean±SE is reported.

A key feature of diabetic vascular lesions is their enhanced vulnerability to rupture, mediated, at least in part, by the generation of procoagulant molecules. To begin to address this concept in murine models, we assessed levels of tissue factor, the key initiator of the procoagulant pathway in vivo. Tissue factor antigen (M₆ ~44 kDa) in the aorta was increased ~1.7-fold by immunoblotting in diabetic versus control tissue (Figure 3). That RAGE was an important mediator of increased levels of tissue factor in the aorta was demonstrated by its suppression to basal levels in diabetic mice treated with sRAGE (Figure 3). Immunohistochemistry localized tissue factor to vascular cells and monocytes in the aorta (Figures V, VI, and VII; please see http://atvb.ahajournals.org). Although these findings do not establish a role for tissue factor in the stability of murine atherosclerotic lesions, they suggest that enhanced generation of tissue factor reflects activation of multiple proinflammatory mechanisms in the vessel wall.

We next sought to determine the molecular mechanisms underlying the enhanced expression of inflammatory mediators in vascular tissue. Previous studies linked the activation of RAGE, by either AGEs or EN-RAGEs, to enhanced nuclear translocation of NF-κB. Compared with nuclear extracts prepared from nondiabetic aorta, diabetic nuclear extracts displayed an ~1.5-fold increase in the activation of NF-κB by EMSA with the use of a radiolabeled consensus probe for NF-κB (Figure 4, lanes 1 to 6 and 7 to 12, respectively). Consistent with an important role for RAGE in mediating enhanced activation of NF-κB in vascular tissue,
Figure 4. Induction of diabetes in apoE-null mice results in enhanced activation of NF-κB in nuclear extracts prepared from aorta: suppression by blockade of RAGE. ApoE-null mice were rendered control or diabetic and treated with sRAGE or MSA. Left, At euthanasia, nuclear extracts were prepared from aorta, and EMSA with use of a radiolabeled consensus probe for NF-κB was performed on nuclear extracts from control mice (lanes 1 to 6), MSA-treated diabetic mice (lanes 7 to 12), and sRAGE-treated diabetic mice (lanes 13 to 18). Lane 19 represents addition of 100-fold excess of unlabeled consensus probe for NF-κB; disappearance of the primary band indicates specificity for NF-κB. Right, Results of densitometric analysis of all bands (mean±SE) are shown.

nuclear extracts prepared from aorta retrieved from sRAGE-treated diabetic mice revealed levels of NF-κB nearly identical to those derived from nondiabetic mice (Figure 4, lanes 13 to 18).

These findings suggested that macrovascular tissue in diabetes displays accelerated inflammation that is, at least in part, due to the activation of RAGE. To determine whether RAGE-mediated proinflammatory events occurred in other tissues susceptible to diabetic complications, we tested the premise that engagement of RAGE and its ligands might mediate cellular activation in kidney tissue.

Compared with levels in nondiabetic mice, levels of RAGE and EN-RAGE antigens were increased ~1.4- and 3.6-fold in diabetic kidney (Figure 5a and 5b, respectively). The receptor was importantly involved in enhancing the expression of these molecules, inasmuch as levels of RAGE and EN-RAGEs were diminished in diabetic mice treated with sRAGE (Figure 5a and 5b, respectively). Immunohistochemistry revealed that EN-RAGEs were expressed largely in inflammatory cells infiltrating diabetic glomeruli (Figure VIII). The observation that increased numbers of EN-RAGE–expressing inflammatory cells were recruited to the diabetic kidney, along with increased expression of VCAM-1 and tissue factor factor, suggested that inflammatory events might alter central properties in the glomerulus. To test this, we performed Northern blotting to assess key cytokines and growth factors implicated in the pathogenesis of diabetic nephropathy. Compared with control kidney tissue, tissue retrieved from diabetic mice displayed an ~1.5-, 2.0-, and 3.8-fold increase in mRNA for transforming growth factor-β, α1 (IV) collagen, and fibronectin (Figure VIII; please see http://atvb.ahajournals.org). In the presence of sRAGE, levels of infiltrating inflammatory cells were significantly reduced, nearly to those levels observed in nondiabetic glomeruli (Figure VIII).

Consistent with the observation that increased numbers of inflammatory cells were identified infiltrating diabetic glomeruli, levels of VCAM-1 antigen were increased ~5.9-fold in diabetic kidneys compared with control kidneys (Figure 6). RAGE was importantly involved in modulating the expression of VCAM-1, inasmuch as an ~44% decrease in levels of VCAM-1 in the kidney was observed in diabetic mice treated with sRAGE (Figure 6). Similar to observations in the aorta, levels of tissue factor antigen in diabetic kidney were increased ~1.5-fold, in a manner suppressed by sRAGE (Figure 7). Immunohistochemistry revealed that compared with nondiabetic or sRAGE-treated diabetic conditions, diabetes was associated with increased expression of tissue factor in the glomerulus and tubular epithelium (online Figures IX, X, and XI; please see http://atvb.ahajournals.org).

The observation that increased numbers of EN-RAGE–expressing inflammatory cells were recruited to the diabetic kidney, along with increased expression of VCAM-1 and tissue factor, suggested that inflammatory events might alter central properties in the glomerulus. To test this, we performed Northern blotting to assess key cytokines and growth factors implicated in the pathogenesis of diabetic nephropathy. Compared with control kidney tissue, tissue retrieved from diabetic mice displayed an ~1.7-, 2.0-, and 3.8-fold increase in mRNA for transforming growth factor-β, α1 (IV) collagen, and fibronectin (online Figures XII, XIII, and XIV, respectively; please see http://atvb.ahajournals.org). In the presence of sRAGE, levels of mRNA for these molecules were suppressed compared with levels observed in nondiabetic controls (Figures XII, XIII, and XIV, respectively).

Levels of glycosylated hemoglobin, serum cholesterol, and triglycerides did not differ between diabetic mice treated with sRAGE versus vehicle, MSA (data not shown).
Figure 7. Induction of diabetes in apoE-null mice results in enhanced expression of tissue factor in kidney: suppression by blockade of RAGE. ApoE-null mice were rendered control or diabetic and treated with sRAGE or MSA. At euthanasia, kidney was retrieved, lysates were prepared, and immunoblotting was performed for tissue factor. The approximate Mₐ of standard molecular weight markers is shown. Representative bands from each condition are shown; immunoblots from 3 mice per condition were performed. Densitometric analysis is indicated, and mean±SE is reported.

Discussion

These findings suggest that induction of diabetes in apoE-null mice accelerated vascular inflammation and expression of tissue factor, as well as the production of glomerular cytoines and extracellular matrix, in a manner that was, at least in part, due to the activation of RAGE. Although previous studies have focused largely on AGEs as signal-transduction ligands of RAGE, the present studies examined the expression of EN-RAGEs, members of the S100/calgranulin family of proinflammatory cytokines, 20, 30 in diabetic tissues. EN-RAGEs, intracellular proteins typically expressed by inflammatory cells, may be released by these cells, thereby enabling them to ligate cellular RAGE and trigger and/or sustain inflammatory pathways. 7 This concept, although well supported in macrovascular disease and atherosclerosis, 11, 32 may stimulate new hypotheses regarding events that set the stage for renal dysfunction. Indeed, previous studies have indicated that S100A mRNA and protein are increased in a range of tissues in experimental type 1 diabetes, including the kidney. 33

Consistent with our findings, studies in Sprague-Dawley rats have demonstrated glomerular macrophage recruitment in early streptozotocin-induced diabetes. 16 The numbers of infiltrating macrophages increased in parallel with enhanced mRNA levels of α₁ (IV) collagen. In irradiated or insulin-treated animals, however, the numbers of recruited macrophages were reduced, again, in parallel with mRNA levels for α₁ (IV) collagen. In the present studies, levels of glucose and glycosylated hemoglobin were unchanged in the presence of RAGE blockade, despite observed decreases in inflammatory cell recruitment and mRNA for extracellular matrix molecules, such as α₁ (IV) collagen and fibronectin. Because this and previous work have indicated that the blockade of RAGE prevents increased expression of the receptor and its ligands in diabetic tissues, our findings suggest that glucose/oxidant stress–mediated generation of AGEs, their interaction with cellular RAGE, and subsequent inflammatory events that, at least in part, involve EN-RAGEs represent critical steps in the cascade of events stimulating cellular activation in diabetic blood vessels and glomeruli. Importantly, examination of human kidney biopsies, compared with age-matched control kidneys, has revealed increased numbers of glomerular mac-

r ophages in mild and moderate glomerulosclerosis, thus suggesting roles for inflammatory events in the pathogenesis of human diabetic nephropathy. 34

Consistent with the concept that RAGE mediates chronic cellular activation in diabetic tissues, nuclear extracts prepared from diabetic aorta, compared with nondiabetic control aorta, displayed enhanced activation of NF-κB by EMSA. In striking contrast to settings such as acute stimulation by lipopolysaccharide or cytokines, for example, in which activation of NF-κB rises rapidly, but transiently, 35, 36 diabetic vasculature demonstrates sustained activation of NF-κB. Although activated NF-κB is evident in euglycemic atherosclerotic lesions, 37 this process appears enhanced in diabetic vasculature, mediated, in large part, by the activation of RAGE.

Taken together, our present data demonstrate that blockade of RAGE in diabetic apoE-null mice suppresses the expression of 2 key mediators of vascular inflammation and activation, VCAM-1 and tissue factor, in parallel with decreased expression of RAGE and EN-RAGEs in aorta and kidney. These findings underscore important roles for ligand-RAGE–mediated inflammatory responses in diabetic tissues and point to unifying molecular mechanisms in the pathogenesis of seemingly diverse complications in the activated diabetic macrovasculature and kidney.

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VCAM-1
Tissue Factor
EN-RAGE(+) cells/glomerules

control  MSA   sRAGE

0.05  0.1  0.15  0.2  0.25  0.3  0.35

c control diabetic

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Tissue Factor