

RECEPTOR FOR AGE (RAGE) IS A GENE WITHIN THE MAJOR HISTOCOMPATIBILITY CLASS III REGION: IMPLICATIONS FOR HOST RESPONSE MECHANISMS IN HOMEOSTASIS AND CHRONIC DISEASE

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1. ABSTRACT

Receptor for AGE (RAGE), a member of the immunoglobulin superfamily, was first identified as a specific cell surface interaction site for Advanced Glycation Endproducts, or AGEs. AGEs, the products of nonenzymatic glycation/oxidation of proteins/lipids, accumulate in natural aging and disorders such as diabetes, renal failure and amyloidoses. Interaction of AGEs with RAGE has been linked to chronic inflammatory and vascular dysfunction that characterizes the chronic complications of these disorders. Recent studies have indicated that RAGE is a multiligand receptor, serving as a specific cell surface, signal transducing receptor for amphoterin, a molecule with implications for neurite outgrowth in neuronal development and in tumor cell proliferation and spread. RAGE is also a receptor for amyloid- β peptide, whose interaction with neuronal and microglial RAGE within the CNS is linked to sustained inflammation and neuronal toxicity and cell death. RAGE also serves as a signal-transducing receptor for EN-RAGEs, and related members of the S100/calgranulin family of proinflammatory cytokines; consequences of this interaction include initiation and propagation of inflammatory responses. Consistent with an important role for ligand-RAGE interaction in these settings, blockade of RAGE suppresses chronic cellular activation and

dysfunction in murine models of diabetic complications, inflammation and tumor proliferation and metastasis. Taken together, a new paradigm is emerging which links RAGE, a gene encoded within the Major Histocompatibility Complex (MHC) Class III regions, to central host response mechanisms in homeostasis and chronic disease.

2. INTRODUCTION

RAGE (Receptor for AGE) is a multiligand receptor of the immunoglobulin superfamily of cell surface molecules (Table 1) first described as a specific cell surface interaction site for Advanced Glycation Endproducts (AGEs), the products of nonenzymatic glycation/oxidation of proteins/lipids (1, 2). AGEs accumulate during normal aging, but to greatly accelerated degrees in disorders such as diabetes, amyloidoses and renal failure. Consistent with the concept that RAGE is a key cell surface receptor for these products, particularly carboxy(methyl) lysine (CML) adducts of proteins/lipids (3), blockade of RAGE in vivo suppresses diabetic vascular hyperpermeability and accelerated atherosclerosis in rodent models (4,5). In addition, recent studies have suggested that RAGE interacts with amyloid- β peptide (A β). Ligand of neuronal and

Table 1. RAGE is a multiligand receptor of the immunoglobulin superfamily of cell surface molecules

Ligand	Physiologic/pathophysiologic conditions
Advanced Glycation Endproducts (AGEs), particularly carboxy(methyl)-lysine adducts EN-RAGEs and molecules	Diabetes, renal failure, aging, ?inflammatory disorders
Amyloid- β -peptide β -cross fibrils	Immune/Inflammatory Disorders, S100/Calgranulin ?Development, Tumors
Amphoterin	Alzheimer's Disease, Amyloidosis Development, Tumors

To date, our studies have identified at least four ligands capable of interacting with RAGE. Activation of RAGE, a signal-transducing receptor for these ligands, appears to play an important role in a range of homeostatic and pathophysiologic conditions.

microglial RAGE in the central nervous system (CNS) by A β has been linked to enhanced oxidant stress, sustained inflammation and neuronal toxicity in Alzheimer's disease (AD) brain (6, 7). More recently, we have identified EN-RAGE (Extracellular Newly-identified RAGE binding protein) (8), and related members of the S100/calgranulin family of proinflammatory cytokines (9, 10), as ligands for RAGE. Engagement of RAGE on endothelial cells, macrophages and lymphocytes activates a cascade of proinflammatory events, resulting in enhanced cellular migration and generation of potent cytokines and growth factors. Importantly, in vivo, blockade of RAGE suppressed the immune/inflammatory response in murine models of delayed-type hypersensitivity (DTH) and colitis in IL-10 null mice (8). In these settings, activation of key cell signalling pathways, such as p21^{ras}, MAP kinases, NF- κ B and cdc42/rac (11 - 13), by RAGE ligand sets the stage for global modulation of cellular properties, in a manner linked to chronic cellular activation and dysfunction.

Studies have suggested that in homeostasis, basal levels of RAGE are expressed in a range of cell types (14). However, a common observation in settings such as diabetic vascular and inflamed tissue, AD brain and immune/inflammatory foci is enhanced expression of RAGE, colocalized with that of ligand. For example, in brain tissue examined from subjects with AD, increased accumulation of A β in neuronal and vascular cellular elements is associated with strikingly increased expression of RAGE, compared with brain retrieved from age-matched controls (6). A clue to the molecular mechanisms underlying these observations exists, at least in part, in the promoter of the gene encoding RAGE. Two functional NF- κ B-like binding elements were identified; site-directed mutagenesis of these NF- κ B binding sites in transfected cells suppressed ligand-mediated regulation of RAGE expression and activation (15). Other potential binding elements, such as NF-IL-6 and γ -interferon response elements identified within the RAGE promoter, may also contribute to regulation of RAGE in immune/inflammatory settings.

The intriguing observation that the gene encoding human RAGE is situated within the Class III

region of the Major Histocompatibility Complex (MHC) (16) ignites further speculation that RAGE responds to a range of stimuli within distinct microenvironments in the organism, driven by the biology of the ligand. Although previous studies suggested a role for RAGE in developmental homeostasis within the CNS (17), it appears likely that RAGE is also intimately linked to pathologic consequences within cells, as its activation by ligand initiates a series of events that portend sustained cellular activation and chronic tissue injury in the host.

3. GLYCOXIDATION, RAGE AND THE PATHOGENESIS OF DIABETIC COMPLICATIONS

Long-term exposure of proteins/lipids to elevated concentrations of aldose sugars, both intra- and extracellularly, accelerates molecular rearrangements of a range of body substrates, leading to the formation and accumulation of the largely irreversible AGEs. Although an heterogeneous class of compounds, composed of such structures as carboxy(methyl) lysine (CML) adducts, pentosidine, pyralline, and methylglyoxal, a dominant AGE observed in vivo is CML-modified protein/lipid adducts (18 - 20). A number of studies indicate that these adducts appear to be in the "right place and at the right time" with respect to diabetic complications, as extent of increased concentrations of tissue CML-modified structures correlated with the degree of complications in diabetic retina and vasculature (21, 22).

3.1. CML AGEs are signal transducing ligands for RAGE

Thus, it was logical to test the hypothesis that engagement of RAGE by CML-AGEs might activate specific cell signalling pathways. Indeed, we found that physiologically-relevant levels of CML modification in proteins bound RAGE on plastic dishes in a dose-dependent manner, with $K_d \sim 76.2 \pm 35$ nM (3), similar to that which was observed with heterogeneous AGEs (61 ± 23 nM) (1). Similar to the effects of in vitro-prepared AGEs and those isolated from the plasma/tissues of diabetic patients (1, 23, 24), CML-modified adducts activated NF- κ B and proinflammatory mechanisms in endothelial cells, macrophages and vascular smooth muscle cells in a RAGE-dependent manner. Infusion of CML-adducts into normal mice increased mRNA and protein for VCAM-1 in lung tissue. That this was dependent on RAGE was demonstrated by experiments in which pretreatment of the mice with anti-RAGE IgG, but not nonimmune IgG, prevented CML-mediated increases in expression of Vascular Cell Adhesion Molecule-1 (3).

Interestingly, formation of CML-adducts is not restricted to the setting of hyperglycemia, as lipid oxidation alone has been suggested to trigger generation of CML AGEs (25). Recent studies indeed suggested that CML-modifications may form as a consequence of activation of the myeloperoxidase-hydrogen peroxide-chloride system, thereby providing a mechanism for the conversion of hydroxy amino acids into glycoaldehyde, an highly-reactive precursor in the steps leading to generation of CML (26).

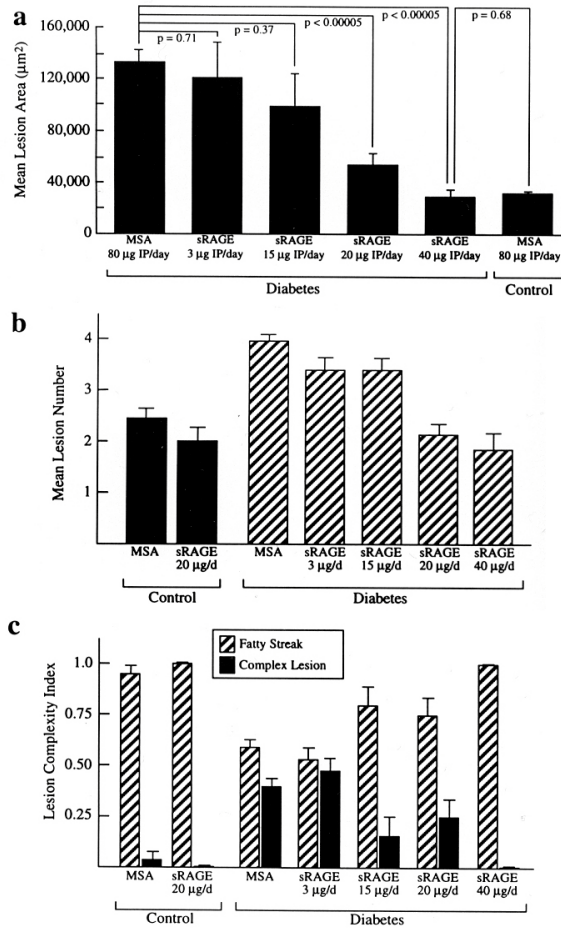


Figure 1. Blockade of RAGE suppresses accelerated diabetic atherosclerosis. **a.** Dose-dependence. Diabetic or control mice were treated as indicated, sacrificed at six weeks, and mean atherosclerotic lesion area determined (μm^2). The results of statistical analysis are shown. There were no statistically-significant differences between diabetic mice and diabetic mice treated with MSA. **b-c.** Effect on lesion number (**b**) and complexity (**c**). **b.** Lesion number. Total lesion number/mouse was determined from analysis of multiple sections prepared at the aortic sinus. Mean total lesion number is reported. Statistical considerations: Diabetes/MSA vs Control/MSA: $p < 0.00005$; Diabetes/MSA vs Diabetes/sRAGE (3 $\mu\text{g/day}$): $p = 0.09$; Diabetes/MSA vs Diabetes/sRAGE (15 $\mu\text{g/day}$): $p = 0.09$; Diabetes/MSA vs Diabetes/sRAGE (20 $\mu\text{g/day}$): $p < 0.00005$; Diabetes/MSA vs Diabetes/sRAGE (40 $\mu\text{g/day}$): $p < 0.00005$; Control/MSA vs Diabetes/sRAGE (20 $\mu\text{g/day}$): $p = 0.30$; and Control/MSA vs Diabetes/sRAGE (40 $\mu\text{g/day}$): $p = 0.12$. **c.** Lesion complexity index. Complexity index was calculated from the ratio of fatty streak (FS)/total lesion number or complex [C] lesion (defined by presence of cholesterol clefts, necrosis or fibrous cap formation)/total lesion number. Therefore, the sum of the ratio of FS/total and C/total is one. Statistical considerations: (Fatty streak index): Diabetes/MSA vs Diabetes/sRAGE 3 or 15 $\mu\text{g/day}$: Not significant; Diabetes/MSA vs Diabetes/sRAGE 20 or 40 $\mu\text{g/day}$: $p < 0.0005$. (Complex lesion index): Diabetes/MSA vs. Diabetes/sRAGE 3 or 15 $\mu\text{g/day}$: Not significant; Diabetes/MSA vs. Diabetes/sRAGE (20 $\mu\text{g/day}$): $p = 0.19$; and Diabetes/MSA vs Diabetes/sRAGE (40 $\mu\text{g/day}$): $p < 0.00005$.

3.2. RAGE and diabetic vasculopathy

A critical complication of diabetes is the premature development and acceleration of atherosclerosis. Multiple studies, including the Diabetes Control and Complications Trial Research Group (DCCT) suggested that although complications of diabetes within the retina and kidney might be modulated by strict control of hyperglycemia, less convincing evidence existed for the protective effects of glucose control in modulating atherogenesis (27). As CML-adducts and RAGE are present in lipid-enriched diabetic atheromata, a critical test of our hypotheses was whether blockade of RAGE might modulate atherogenesis in experimental models. Since mice, innately, are highly resistant to the development of aggressive, complex atherosclerosis, even in the presence of lipid-enriched diets and diabetes (28), we tested these concepts in mice genetically-primed for the development of atherosclerosis. In our first studies, we employed mice deficient in apolipoprotein E (29, 30), a model of spontaneous hypercholesterolemia and aggressive arterial lesion formation. Induction of diabetes in these mice, by serial administration of streptozotocin, resulted in an ~5.3-fold increase in mean atherosclerotic lesion area at the aortic sinus (5). Atherosclerotic lesions in diabetic apo E null mice, enriched in CML-AGEs, demonstrated increased expression of vascular RAGE compared with age-matched nondiabetic mice (5).

To test the hypothesis that blockade of RAGE might modulate atherogenesis in diabetic mice, we prepared soluble RAGE (sRAGE). The extracellular domain of RAGE is composed of one "V"-type Ig domain, followed by two "C"-type Ig domains, and is the site within the molecule which engages ligand (specifically within the V-domain (1, 5)). Based on the kinetics of this molecule *in vivo* (4), murine recombinant sRAGE was administered once daily to apo E null mice, immediately upon documentation of diabetes. Intraperitoneal injection of sRAGE induced dose-dependent suppression of mean atherosclerotic lesion area, number and complexity at the aortic sinus (Figure 1A-b-c, respectively). Importantly, the effects of sRAGE occurred independently of alterations in plasma glucose, insulin or lipid number/profile (5), thereby highlighting the importance of CML (AGE)-RAGE interaction in the development of accelerated diabetic atherosclerosis.

In addition to suppression of atherosclerosis, our findings suggested that blockade of RAGE diminished levels of oxidant stress *in vivo*, as demonstrated by increased lag time to copper-induced oxidation of LDL from sRAGE-treated diabetic mice, as well as decreased levels of plasma and tissue AGEs compared with control apo E null mice (5). Overall, we speculate that diminished CML (AGE)-RAGE interaction limited generation of further reactive oxygen intermediates, thereby interrupting a chronic cascade of cellular activation, enhanced oxidant stress and increased inflammatory gene expression.

Recent studies have extended these findings to other models of diabetic complications. For example, blockade of RAGE suppressed accelerated alveolar bone loss and periodontal inflammation in diabetic mice infected with the human periodontal pathogen, *Porphyromonas gingivalis* (31), thus highlighting RAGE as an important pathogenic factor in the host response to bacterial infection in diabetes.

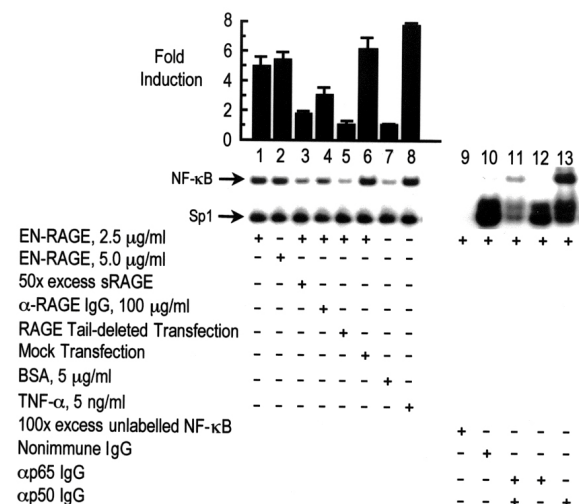


Figure 2. Ligation of RAGE by EN-RAGE activates endothelial cells (electrophoretic mobility shift assay). Human umbilical vein endothelial cells (HUVEC) were treated with the indicated mediators for eight hrs. In certain cases, EN-RAGE-treated cells were preincubated with anti-RAGE IgG and in other cases, EN-RAGE was pretreated with excess sRAGE (two hours). Certain HUVEC were transiently-transfected with a construct encoding a form of human RAGE in which the cytosolic domain was deleted or with vector alone as control (pcDNA3) prior to treatment with EN-RAGE. Nuclear extract was prepared and EMSA performed employing radiolabelled probes for NF-κB and Sp1. Supershift assays were performed by incubation of nuclear extract with the indicated antibody prior to EMSA. Results of densitometric analysis after normalization for Sp1 are indicated. Supershift assays were performed by incubation of nuclear extract with the indicated antibody (2 µg/ml) for 45 minutes prior to EMSA. Results of densitometric analysis are indicated in the figures.

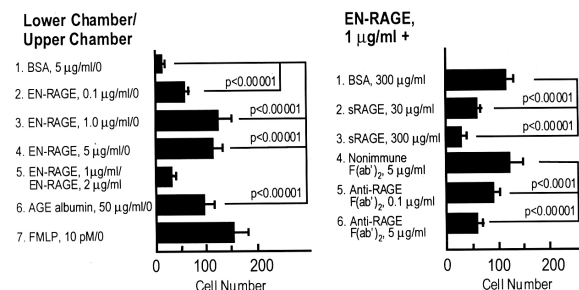


Figure 3. Ligation of RAGE by EN-RAGE activates peripheral Derived mononuclear phagocytes (MPs) (modified chemotaxis assays). Mediators were placed in the upper or lower chamber, and human peripheral blood-derived monocytes placed in the upper chamber for 4 hrs. Cells which had migrated through the membranes were stained and counted (left panel). Where indicated (right panel), cells were pretreated with the indicated F(ab')₂ fragments or EN-RAGE incubated with excess sRAGE prior to chemotaxis assay. Mean ± SD is shown.

4. EN-RAGES, RAGE AND THE INFLAMMATORY RESPONSE

An important challenge in the study of RAGE was the quest for "natural ligands" of the receptor, as

products of glycooxidation of proteins/lipids were unlikely to represent the sole ligand for a receptor in the immunoglobulin superfamily. In order to identify molecules that might interact with RAGE beyond AGEs, we returned to the site in which the receptor itself was first identified: lung tissue. Using a series of chromatographic steps, and ultimately purification on resin to which had been adsorbed sRAGE, we identified two polypeptides based on their ability to bind RAGE. The first, amphoterin, was a polypeptide identified with high levels in developing neurons of the CNS and neurite outgrowth in vitro (17, 32, 33); and the second was a polypeptide of the S100/calgranulin family of proinflammatory cytokines, S100A12 or calgranulin C (8). The latter molecule was first denoted as EN-RAGE, since at the time of its first isolation from lung tissue, its sequence had not yet been reported to the data banks. In 1996-1997, the sequence of the molecule was published by two groups (34-36). EN-RAGE and S100/calgranulin polypeptides bound immobilized RAGE in a dose-dependent manner, with $KD \sim 91 \pm 29$ nM. Specificity of binding was shown by inhibition in the presence of excess soluble RAGE and by other ligands for the receptor, including AGE albumin, amphoterin and Aβ (8). The latter findings suggest that the ligands, although seemingly diverse in structure, appear to interact with a common site/element within RAGE. Studies are underway to elucidate the structure of RAGE as a means to further clarify our understanding of ligand-receptor interactions.

As RAGE was present on a number of cell types, we sought evidence for its interaction with EN-RAGEs in cells importantly involved in the inflammatory response.

4.1. Endothelial cells

Incubation of EN-RAGEs with RAGE on cultured EC resulted in dose-dependent increases in expression of VCAM-1, thereby providing a mechanism for targeting mononuclear inflammatory cells to activated EC. Enhanced expression of VCAM-1 was associated with increased function of this molecule, as EN-RAGEs stimulated increased adherence of Molt-4 cells, which bear the counterligand for VCAM-1, to stimulated EC in a RAGE-dependent manner (8). A central mechanism underlying EN-RAGE-RAGE-mediated upregulation of inflammatory response genes such as VCAM-1 was elucidated by studies in which incubation of EC with EN-RAGEs resulted in an ~5-fold increase in nuclear translocation of NF-κB by electrophoretic mobility shift assay (EMSA) (Figure 2). That these effects were largely mediated by EN-RAGE with cell surface RAGE was demonstrated by the inhibitory effects of anti-RAGE IgG or sRAGE. Importantly, RAGE-dependent engagement of intracellular cell signalling mediators was essential for activation of NF-κB, as introduction of a form of RAGE lacking the cytosolic domain (but firmly anchored in the membrane via the extracellular/transmembrane spanning domains), exerted a dominant negative (DN) effect (Figure 2).

Since EN-RAGEs bear homology to other members of the S100/calgranulin family, we tested the effects of another member, S100B, in activation of NF-κB. Incubation of HUVEC with human S100B resulted in an ~3.2-fold increase in nuclear translocation of NF-κB (8). These effects were inhibited in the presence of sRAGE,

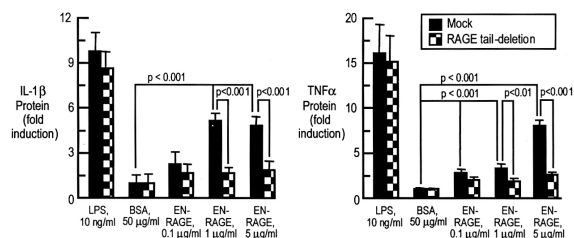


Figure 4. Ligation of RAGE by EN-RAGE activates mononuclear phagocytes (generation of IL-1 β and TNF- α). BV-2 macrophages, either those transfected with DN-RAGE or mock-transfected cells, were incubated with the indicated mediators for 8 hrs at 37°C. Supernatant was collected and ELISA for IL-1 β or TNF- α performed. Results are reported as fold X1 induction, compared with incubation of cells with BSA alone. Mean \pm SD is shown.

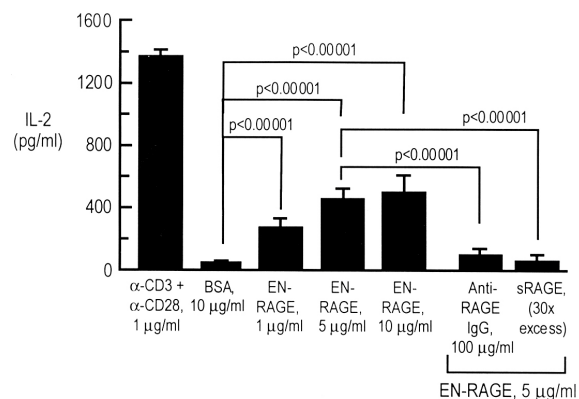


Figure 5. Ligation of RAGE by EN-RAGE activates PBMCs (generation of IL-2). PBMC were incubated with the indicated mediators for 8 hrs; supernatant was collected and ELISA for IL-2 performed. Where indicated, cells were pretreated with the indicated IgG, or EN-RAGE was pretreated with excess sRAGE. Mean \pm SD is reported. Results are reported as fold induction (incubation of cells with BSA alone).

anti-RAGE IgG or introduction of DN RAGE. Thus, a range of S100/calgranulin polypeptides likely interact with RAGE.

4.2. Mononuclear Phagocytes

Since MPs are a rich source of EN-RAGEs, we postulated that once recruited to sites of inflammation, their release by such cells might provide a mechanism for further cellular activation, by interaction with RAGE. Indeed, studies in modified chemotaxis chambers using human peripheral blood-derived monocytes, indicated that EN-RAGEs induced monocyte migration in a dose- and RAGE-dependent manner (Figure 3, left and right panels). Similarly, engagement of RAGE by EN-RAGEs in cultured Bv2 cells (murine macrophages), induced elaboration of both IL-1 β and TNF- α into cellular supernatants; effects which were suppressed by introduction of DN-RAGE into these cells (Figure 4, left and right panels, respectively). In view of the ability of EN-RAGEs to enhance proinflammatory cytokine expression in monocytes, we assessed the role of NF- κ B in these

processes. EN-RAGEs were a strong agonist for induction of nuclear translocation of NF- κ B via RAGE, as demonstrated by EMSA (8).

4.3. Peripheral Blood Mononuclear Cells (PBMCs)

Our findings in EC and MPs strongly suggested that engagement of RAGE by EN-RAGEs was a potent mechanism for propagation of proinflammatory signals at sites of immune/infected foci. To extend these findings to PBMC, cells critically involved in immune/inflammatory mechanisms, we demonstrated for the first time that RAGE was indeed present on these cells (8). PBMCs exposed to EN-RAGEs displayed enhanced elaboration of IL-2 into supernatant, in a RAGE-dependent manner, as these effects were suppressed in the presence of sRAGE or anti-RAGE IgG (Figure 5). Consistent with these findings, an enhanced mitogenic response to cross-linking CD3/CD28 after stimulation with EN-RAGEs was noted in PBMCs. Compared with pretreatment with albumin, significant uptake of 3 H-thymidine was observed in cells preincubated with EN-RAGE (5 μ g/ml), 39,285 \pm 2,323 vs 67,242 \pm 1,727 counts per minute, respectively; $p < 0.00001$ (8).

These studies, therefore, in cultured EC, MPs, and PBMCs, strongly implicated a role for EN-RAGE-RAGE interaction in cells critical for initiation and propagation of the inflammatory response as this interaction modulated central properties of cell migration, proliferation and cytokine generation.

4.4. EN-RAGE-RAGE interaction: in vivo models

To test these concepts in vivo, EN-RAGEs were infused intravenously into immune-competent mice. Examination of lung tissue revealed increased expression of VCAM-1 protein; this effect was inhibited by pretreatment with anti-RAGE IgG or excess sRAGE (8). Injection of EN-RAGEs into murine foot pad resulted in increased inflammation, as assessed by scoring of clinical foot pad redness, swelling and edema, and by histologic scoring, using hematoxylin and eosin (8). Consistent with an important role for RAGE in this milieu, compared with treatment with vehicle, murine serum albumin (MSA) or nonimmune F(ab') $_2$, those mice pretreated with sRAGE or anti-RAGE/anti-EN-RAGE F(ab') $_2$ demonstrated marked suppression of inflammatory score after local injection of EN-RAGE into the foot pad (8).

The critical tests of these concepts was in models of acute/chronic inflammation. Murine models of delayed-type hypersensitivity provided an ideal setting with which to test the role of RAGE blockade in suppression of inflammatory responses. To test this, methylated BSA (mBSA; not a ligand for RAGE) was injected in the regional groin lymph nodes of CF-1 mice in the presence of incomplete Freund's adjuvant. Three weeks later, mice were locally-challenged with mBSA by footpad injection. Mice pretreated with sRAGE or anti-RAGE/anti-EN-RAGE F(ab') $_2$ demonstrated marked suppression of inflammation score compared with mice receiving vehicle (Figure 6). Diminished activation of NF- κ B, determined

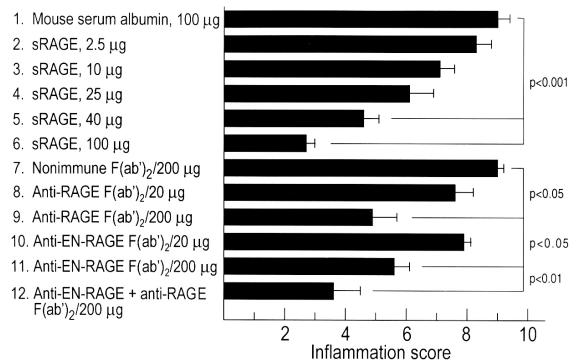


Figure 6. Blockade of EN-RAGE/RAGE suppresses inflammation in a model of delayed-type hypersensitivity (DTH). CF-1 mice were sensitized and challenged with methylated BSA. Mice were pretreated by intraperitoneal injection with sRAGE, MSA, immune or nonimmune F(ab')₂ fragments prior to and after local challenge with mBSA. 24 hrs after injection of foot pad with mBSA, clinical and histologic score of foot pad was performed employing the following criteria: Inflammation score (maximal of 9; no inflammation = 2) is defined as the sum of the clinical and histologic score: Clinical score: 1=absence of inflammation; 2= slight rubor and edema; 3=moderate rubor and edema with skin wrinkles; 4= severe rubor and edema without skin wrinkles; and 5=severe rubor and edema with toe spreading. Histologic score (H&E studies): 1=no leukocytic infiltration or subcutaneous edema; 2=slight perivascular leukocytic infiltrate with slight subcutaneous edema; 3=severe leukocytic infiltrate without granulomata; and 4= severe leukocytic infiltrate with granulomata. Mean \pm SD of n=3/group is shown.

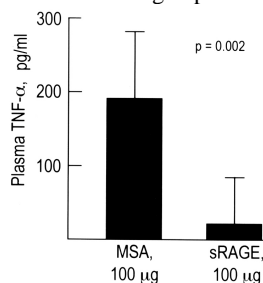


Figure 7. Blockade of EN-RAGE/RAGE suppresses chronic colonic inflammation in IL-10 null mice. Assessment of plasma TNF- α . Plasma was retrieved from IL-10 null mice treated with either six weeks sRAGE, 100 µg once daily, or vehicle, murine serum albumin (MSA) upon sacrifice and ELISA for TNF- α performed. Mean values for MSA- (n=6) vs sRAGE-treated mice (n=6) were 190.5 ± 89.0 vs 21.9 ± 63.6 pg/ml, respectively; $p=0.002$.

by EMSA using nuclear extract prepared from foot pad tissue, was noted in the presence of RAGE/EN-RAGE blockade, as was decreased production of foot pad TNF- α (8).

Lastly, we tested these concepts in mice deficient in IL-10, a model of spontaneous inflammatory bowel disease (IBD) (37). Indeed, levels of EN-RAGEs/S100 calgranulin molecules were associated with degree of inflammation in human IBD (38). We thus tested the role

of RAGE blockade in this murine model. Compared with treatment with vehicle, MSA, administration of sRAGE resulted in decreased evidence of rectosigmoid inflammation (8). Consistent with this observation, a marked suppression of activation of NF- κ B in nuclear extracts prepared from rectosigmoid tissue was observed in sRAGE-treated mice (8) compared with vehicle. Similarly, decreased levels of plasma TNF- α were noted in IL-10 null mice exposed to RAGE blockade (Figure 7).

Although future studies will be necessary in transgenic mice with modified levels of RAGE/RAGE function, and in mice deficient in RAGE, the present work provides new insights into important pathogenic roles for EN-RAGE molecules and RAGE in inflammatory responses.

5. AMPHOTERIN AND RAGE: INSIGHTS INTO NEURONAL DEVELOPMENT AND TUMOR BIOLOGY

As noted earlier, the search for putative natural ligands for RAGE led not only to identification of EN-RAGEs, but also to amphoterin. Amphoterin, or high mobility group 1 protein, was previously demonstrated to mediate neurite outgrowth of CNS neurons retrieved from day 17 rat embryos (32, 33). The increased expression of RAGE in the developing CNS, which co-localized with that of amphoterin, strongly suggested that amphoterin-RAGE interaction might be important in neuronal development. Yet, in both cases, expression of these molecules was significantly diminished in neurons retrieved on postnatal day 5. These considerations suggested that the temporal expression of these molecules in development was linked to a specific function.

5.1. Amphoterin-RAGE interaction induces neurite outgrowth

Consistent with this hypothesis, previous studies demonstrated that amphoterin bound RAGE in a dose-dependent manner, on both plastic dishes and on cultured E17 cortical neurons (17). Importantly, treatment of neurons with sRAGE or anti-RAGE F(ab')₂ suppressed neurite outgrowth *selectively* on amphoterin-coated matrices. Blockade of RAGE had no suppressive effect on neurite outgrowth on poly-L-lysine or laminin-coated dishes (17). These findings suggested the importance of amphoterin-RAGE interaction in mediating central cellular properties of invasion/migration. Certainly, however, definitive evidence for a role for amphoterin-RAGE will lie in studies employing mice in which these molecules have been genetically-deleted.

Indeed, the observation that amphoterin and RAGE are expressed in tumor cells, especially at the leading edge of advancing processes, suggested their contribution to tumor invasion and spread. Recent observations suggest a role for this ligand/receptor interaction in tumor properties since blockade of RAGE/amphoterin suppressed local growth of C6 glioma and metastases of Lewis lung carcinoma in murine models (39, 40). Future studies will address this axis fully,

especially with respect to cell signalling mechanisms that underlie the roles of these molecules in tumor properties.

5.2. Amphoterin: a possible role in sepsis and cellular injury

Thus, although amphoterin appears to have homeostatic function, at least in development, it is likely that pathologic effects of amphoterin-RAGE interaction in other settings may exist. Beyond a potentially-pathogenic role in tumor biology, recent studies have suggested that amphoterin may be released by lipopolysaccharide (LPS)-stimulated macrophages 8-32 hours after exposure, thereby exerting an effect as a late mediator of morbidity/mortality in mice to whom lethal doses of LPS were administered. The observation that administration of blocking antibodies to amphoterin protected mice from otherwise lethal septicemia strongly suggests that engagement of cell surface receptors, such as RAGE, might importantly mediate the pathogenic effects of amphoterin (41). Studies are underway determine if amphoterin-RAGE interaction may represent an additional means of sustaining inflammatory responses and tissue injury in sepsis, as injection of amphoterin protein imparted lethal effects (41).

6. AMYLOID- β PEPTIDE AND NEURONAL TOXICITY

A dichotomy between likely roles for RAGE in homeostasis and in pathologic states is emerging, especially in the nervous system. A search for cell surface interaction sites for A β in extract of bovine lung uncovered two bands, ~50 and 30-35kDa. Amino-terminal sequence analysis revealed that both contained sequences for RAGE, the latter likely the extracellular domain, cleaved from full-length forms after proteolysis (6). Further study elucidated enhanced expression of RAGE, co-localizing with that of A β in human AD brain tissue, in neurons, microglia and vascular elements (6). Suggestive of an important role for A β -RAGE interaction in neuronal toxicity, A β bound RAGE in a dose-dependent manner, on plastic dishes and on RAGE-transfected cos cells (6). Indeed, in cell culture models, incubation of A β with RAGE-transfected cos cells induced increased generation of thiobarbituric acid reactive substances (TBARS), and activation of NF-kB; these observations were suppressed in the presence of anti-RAGE IgG or sRAGE (6). Furthermore, A β activated microglia via RAGE, as evidenced by increased microglial migration and generation of TNF- α mRNA and protein (6). These findings thus provided a mechanism for *direct* A β -induced neuronal toxicity, as well as a means to enhance inflammation within A β -enriched CNS elements, by induction of inflammatory, neurotoxic mediators by activated microglia. Consistent with this concept, further study indicated that incubation of A β with neuronal cells resulted in increased generation of macrophage-colony stimulating factor (m-csf), via RAGE-dependent oxidant/NF-kB-requiring pathways, thus providing a mechanism for enhanced proinflammatory events in an A β -enriched setting such as AD (7). Experiments are underway in transgenic mice overexpressing both neuronal A β and RAGE to rigorously test these concepts in vivo.

7. CONCLUSIONS

An emerging view of the MHC Class III region suggests that genes encoded within this site generate products importantly involved in host responses to a range of innate and environmental cues, both in homeostasis and disease. In parallel, our developing understanding of the biology of RAGE suggests that it participates in a number of host-modifying mechanisms, likely, at least in part, in response to varying temporal and spatial localization of its ligands, particularly in specific microenvironments.

Multiple lines of evidence suggest that in situations characterized by increased accumulation of RAGE ligand, the expression of the receptor, rather than undergoing down-regulation in response, is, in fact, enhanced. Likely underlying molecular mechanisms include engagement of binding elements for NF-kB within the RAGE promoter, as ligand such as AGEs, A β and EN-RAGEs activate NF-kB; one consequence of which is activation of proinflammatory mechanisms, including upregulation of RAGE expression itself (15). Furthermore, we recently described that enhanced expression of RAGE in neuronal-like cells may be effected via amphoterin; amphoterin-RAGE interaction prompts nuclear translocation of Sp1, thereby increasing transcription of mRNA for RAGE (42). These considerations provide a mechanism for cellular activation driven, at least in part, by properties of RAGE ligand.

We speculate that in the presence of limited expression of ligand, such as amphoterin in developing brain, RAGE may serve a homeostatic role. The striking decreases in amphoterin/RAGE expression in CNS neurons by day 5 of development suggest that a critical function, such as cellular spreading and neurite outgrowth, was largely accomplished. However, in the presence of chronic, sustained levels of amphoterin in rapidly-growing tumors, for example, sustained activation of RAGE may imbue pathologic consequences of dysregulated cell growth and invasion, properties essential for local tumor growth and distant spread.

Thus, although a seemingly diverse group of structures, the ligands of RAGE identified, at least to date, appear to share a common binding site within the extracellular domain of RAGE, especially within the V-type Ig domain. Studies are underway to elucidate the crystal structure of RAGE, both alone and in the presence of ligand, in order to enhance our understanding of the precise conditions necessary for activation of RAGE.

In this context, recent studies have delineated a polymorphism within the V-domain of RAGE; in that form of the molecule, glycine encoded at position 82 is converted to serine (Gly82Ser) (43). In one study, this polymorphism was identified in 10% of Asian subjects examined, and in 12% of Caucasian individuals (43). Although at first glance, at least, the presence of the polymorphism did not appear to be associated with the incidence of *macrovascular* complications in the diabetic subjects, other studies suggested a possible relationship to

skin complications in type 2 diabetes (44) and in microangiopathy (45).

Indeed, the recent intriguing report of a strong linkage disequilibrium between the variants of RAGE carrying the serine amino acid at position 82, and two HLA-DR2 and HLA-DR4 specificities with the HLA class II region (44, 46) are consistent with these observations, since DR4 specificity has been linked previously to diabetic microangiopathy (47, 48). Furthermore, potential links between this serine variant of RAGE and susceptibility to immune-related diseases and/or extent of chronic cellular activation and tissue injury in settings characterized by increased expression/accumulation of RAGE and its ligands, CML-AGEs, EN-RAGEs, amphoterin, and A β , represent an exciting area for future investigation.

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