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Review

RAGE is a multiligand receptor of the immunoglobulin superfamily: implications for homeostasis and chronic disease

L. G. Bucciarelli, T. Wendt, L. Rong, E. Lalla, M. A. Hofmann, M. T. Goova, A. Taguchi, S. F. Yan, S. D. Yan, D. M. Stern and A. M. Schmidt*

College of Physicians & Surgeons, Columbia University, 630 W. 168th Street, P&S 17-401, New York, NY 10032 (USA), Fax: + 1212 305 5337, e-mail: ams11@columbia.edu

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Abstract. Receptor for AGE (RAGE) is a member of the immunoglobulin superfamily that engages distinct classes of ligands. The biology of RAGE is driven by the settings in which these ligands accumulate, such as diabetes, inflammation, neurodegenerative disorders and tumors. In this review, we discuss the context of each of these classes of ligands, including advance glycation endproducts, amyloid β peptide and the family of β sheet fibrils, S100/calgranulins and amphoterin. Implications for the role of these ligands interacting with RAGE in homeostasis and disease will be considered.

Key words. Receptor; diabetes; vasculopathy; atherosclerosis; immunoglobulin; superfamily; inflammation; cancer.

Introduction

Receptor for AGE (RAGE) was first identified as a cell surface receptor for the products of nonenzymatic glycation and oxidation of proteins, the advanced glycation endproducts (AGEs) [1, 2], adducts that accumulate in settings such as diabetes, renal failure and aging. Although AGEs are a heterogenous group of structures, the most prevalent class of AGEs found in vivo, N^{ε} (carboxymethyl)lysine (CML) adducts, are signal transduction ligands of RAGE [3–7]. The finding that CML-modified adducts accumulate in settings of oxidant stress, such as inflammatory milieu, highlighted the strong possibility that the biology of RAGE was not limited to diabetes and its complications. Indeed, subsequent studies suggested that RAGE, a member of the immunoglobulin superfamily,was unlikely to have evolved solely to transduce cellular signals triggered by AGEs [8]. A search for putative natural ligands of the receptor uncovered unexpected results. In addition to AGEs, RAGE is a signal transduction receptor for amyloid-β peptide (Aβ) and β sheet fibrils [9], S100/calgranulins, members of a family of proinflammatory cytokines [10] and amphoterin, a molecule linked to neurite outgrowth and cellular motility, especially in tumors [11]. Work is ongoing to explore the molecular and cellular consequences of these ligand-receptor interactions in both health and disease.

Diabetes and its complications

Many studies have rigorously demonstrated that the incidence and severity of atherosclerosis are increased in human subjects with diabetes [12–14]. The work of the Diabetes Control Clinical Trials (DCCT) group suggested that the complications of diabetes do not ensue solely from the effects of hyperglycemia itself [15]. Of course, the direct effects of hyperglycemia have been shown to include activation of protein kinase C (PKC), especially the β isoforms, and enhanced nuclear translocation of NF-κB, a central transcription factor involved in evolution of the inflammatory response [16–21]. Together, such observations suggest that elevated levels of glucose engaging the vasculature may impart signals linked to cellular activa-

* Corresponding author.

tion and, long term, tissue dysfunction. In addition, however, the results of the DCCT suggest that indirect consequences of high levels of blood sugar may contribute importantly to the pathogenesis of complications in diabetes. In this context, hyperglycemia, alone or in parallel with enhanced oxidative stress, leads to the formation of AGEs. AGEs are a heterogeneous class of structures, such as CML, pentosidine and pyralline [22–33]. CML adducts, in particular, and the most prevalent AGEs identified thus far in vivo, are signal transduction ligands of RAGE [7]. In in vitro and in vivo studies, we found that physiologically relevant concentrations of CML adducts activate endothelium (EC), vascular smooth muscle cells (VSMCs) and macrophages (MPs) and lead to expression of a range of proinflammatory molecules. Activation of NF-κB is a key pathway leading to such events. The central role of RAGE in mediating the pathogenic effects of AGEs such as CML was shown by (i) suppression of cellular activation in the presence of soluble RAGE, the extracellular ligand-binding domain of RAGE which acts as a decoy to trap ligand and prevent interaction with, and activation of, cell surface receptor, (ii) blocking antibodies to RAGE or (iii) introduction of a form of RAGE in which the cytosolic domain was deleted, thereby rendering cells capable of binding ligand, but unable to mediate intracellular signalling, a 'dominant negative' (DN) effect [7]. One should note that other cell surface interaction sites for AGEs have been identified, such as macrophage scavenger receptor (MSR), and AGE-1, -2 and -3 [34–36]. However, none of these binding sites has been shown to mediate cell signalling induced by AGEs; rather, these interaction sites, distinct from RAGE, may serve to remove AGEs from the extracellular milieu and, potentially, detoxify them. Our studies have focussed on RAGE as a signal transduction receptor for these structures.

The accumulation of AGEs, and in particular CML-modified adducts, in diabetic tissues is not surprising. Importantly, we and others have demonstrated that although RAGE is present at low, homeostatic levels in the tissues, its expression is enhanced in diabetes. Expression of RAGE is increased in an overlapping manner with AGES in cells such as EC and smooth muscle, inflammatory cells such as monocytes, and in podocytes of the kidney [37–41]. Thus, instead of down-regulating expression of RAGE, these ligands may contribute to *enhanced* expression of the receptor in the diabetic milieu. Consistent with this concept, the promoter of the gene encoding RAGE contains at least two functional NF-κB elements; these sites, responsive to proinflammatory stimuli such as lipopolysaccharide (LPS) and tumor necrosis factor-α (TNF-α), may be responsible, in part, for the increased expression of RAGE observed at sites of ligand accumulation [42]. These concepts led us to hypothesize that a smoldering degree of cellular activation results from chronic AGE-RAGE interaction in diabetic tissues. Upon superimposition of biologic or physical stress, such as bacterial infection, disruption of the tissues by wounding or increased levels of lipids, an augmented host response occurs selectively in the diabetic environment, thereby leading to enhanced cellular activation and exaggerated tissue injury. Such a 'two-hit' model of RAGE-mediated cellular stress may explain, in part, complications of diabetes such as those in infected periodontium, impaired wound healing and accelerated atherosclerosis.

To dissect the contribution of ligand/RAGE interaction in the pathogenesis of diabetic complications, we have tested these concepts in vivo using reagents to block the receptor itself, or have blocked access to RAGE by administering a decoy, soluble or (s)RAGE. In our first studies, we tested the role of blockade of RAGE in suppressing diabetes-associated hyperpermeability, a well-established surrogate marker for diabetic vasculopathy in rats rendered diabetic with streptozotocin (stz) [43–46]. First, in euglycemic animals, infusion of AGE-bearing diabetic red blood cells from syngeneic rats resulted in increased permeability in a number of organs, as assessed by the tissue-blood isotope ratio (TBIR). These effects were suppressed in the presence of blockade of RAGE, using either sRAGE or monospecific antibodies blocking the receptor [47]. Furthermore, after 9–11 weeks of diabetes, diabetic rats displayed increased permeability (TBIR) in multiple organs. Infusion of sRAGE into these animals immediately prior to determination of TBIR revealed near normalization of tissue permeability by blockade of RAGE [47]. These experiments, the first to show that blockade of RAGE in vivo might affect an established complication of diabetes, led the way to our study of blockade of RAGE in models of *chronic* complications of diabetes that have been developed in murine models.

Diabetes-associated hyperpermeability was especially important for our study of diabetic vascular disease, because increased permeability in human subjects with diabetes is associated with increased morbidity and mortality from cardiovascular complications [45, 46]. Thus, we sought to test our hypotheses in a murine model of atherosclerosis. Mice are inherently resistant to the development of complications, because their lipid profile, enriched in functional high-density lipoprotein (HDL) largely protects them from the adverse effects of diet-associated fat intake. To overcome this, we tested these concepts in mice already susceptible to the development of atherosclerosis. In apo E null mice rendered diabetic with stz and maintained on a normal chow diet, induction of diabetes was associated with an approximately fivefold increase in mean atherosclerotic lesion area at the aortic sinus after 6 weeks of diabetes compared with euglycemic apo E null mice of the same age [39]. Diabetes-associated atherosclerotic lesions in this model displayed increased accumulation of AGEs and enhanced expression of RAGE. Importantly, in addition to lesion area, le-

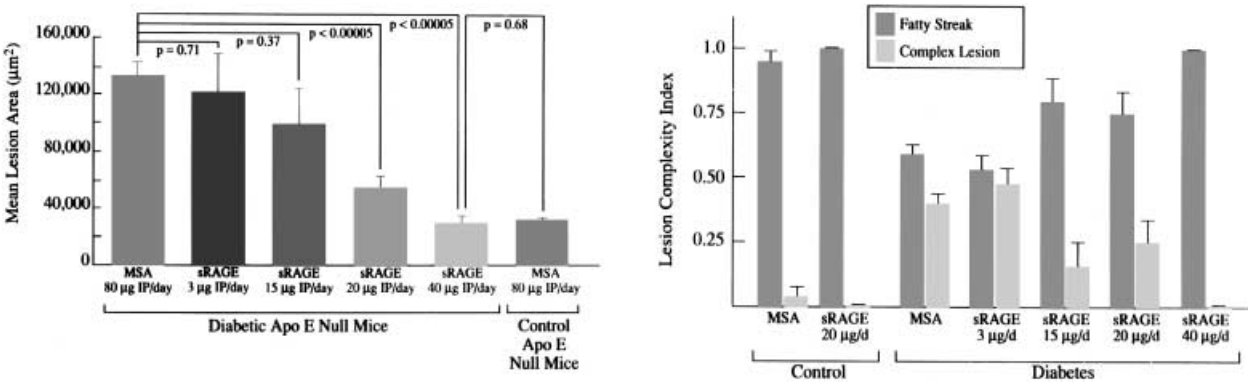

Figure 1. sRAGE suppresses accelerated diabetic atherosclerosis. (*Left*) Lesion area. Diabetic or control mice were treated as indicated, sacrificed at 6 weeks, and mean atherosclerotic lesion area determined (μm^2). The results of statistical analysis are shown. There were no statistically significant differences between diabetic mice and diabetic mice treated with murine serum albumin (MSA). (*Right*) Lesion complexity. A 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. Ratios were as follows: control/MSA: FS = 0.95 ± 0.04, C = 0.05 ± 0.04; control/sRAGE (20 μg/day): FS = 1 ± 0, C = 0 ± 0; diabetes/MSA: FS = 0.59 ± 0.04, C = 0.41 ± 0.04; diabetes/sRAGE (3 μg/day): FS = 0.54 ± 0.06, C = 0.46 ± 0.06; diabetes/sRAGE (15 μg/day): FS = 0.84 ± 0.10, C = 0.16 ± 0.10; diabetes/sRAGE (20 μg/day): FS = 0.75 ± 0.09, C = 0.25 ± 0.09; diabetes/sRAGE (40 μg/day): FS = 1 ± 0, C = 0 ± 0. Statistical considerations – FS index: diabetes/MSA vs diabetes/sRAGE 3 or 15 μg/day, not significant; diabetes/MSA vs diabetes/sRAGE 20 or 40 μg/day, $p < 0.0005$; C index: diabetes/MSA vs diabetes/sRAGE 3 or 15 μg/day, not signifi-cant; diabetes/MSA vs diabetes/sRAGE (20 μg/day), $p = 0.19$; diabetes/MSA vs diabetes/sRAGE (40 μg/day), $p < 0.00005$.

sion complexity was accelerated in this model. Complex lesions, defined as cholesterol clefts, necrosis or fibrous caps were much more commonly observed in diabetic mice at age 14 weeks versus controls [39]. To test the hypothesis that activation of RAGE contributed, at least in part, to these observations, we treated diabetic apo E null mice with once daily injections of murine sRAGE immediately upon documentation of diabetes. Administration of sRAGE suppressed accelerated lesion area and complexity in a dose-dependent manner (fig. 1). At the highest dose of sRAGE tested, atherosclerotic lesion area size and complexity did not differ from that observed in euglycemic apo E null mice of the same age [39]. In parallel with these observations, we found that vascular tissue factor, VCAM-1, AGEs and nuclear translocation of NF-κB were decreased in sRAGE-treated mice compared to vehicle-treated diabetic animals [48]. The effects of blockade of RAGE were independent of changes in blood sugar level or lipid number/profile. These observations demonstrated that AGE-RAGE interaction represents a central pathway linked to acceleration of atherogenesis in diabetes. Similar results were observed in other murine models of hyperlipidemia; for example, induction of diabetes in LDL receptor null mice resulted in accelerated atherosclerosis, a process prevented by adminsitration of sRAGE [49]. Furthermore, these concepts are applicable in murine models of insulin-resistant (type 2) diabetes. In recent experiments, we bred apo E null mice into the db/db background. Apo E null/db/db mice displayed markedly accelerated atherosclerosis at the aortic sinus, along with strikingly increased vascular inflammation. These effects were prevented by administration of sRAGE, commencing immediately at the time of documentation of diabetes at age 8 weeks [50].

A central extension of these observations is the degree to which blockade of RAGE might impact on *established* atherosclerotic plaques. Certainly, if these concepts are to be translated to the clinical milieu, demonstrating that in long-standing diabetes, intervention by blockade of RAGE may impact on already developed lesions will be essential. To study this, apo E null mice were rendered diabetic at age 8 weeks. Mice were untreated until age 14 weeks; at that time, treatment was begun for an additional 6 weeks with either sRAGE, or vehicle, murine serum albumin (MSA). Administration of sRAGE suppressed progression of atherosclerotic lesion area and complexity, thereby indicating that ongoing inflammatory processes within the lesions that serve to render the lesions more susceptible to instability may be impacted by blockade of RAGE [51, 52]. These considerations suggested that RAGE is a progression/amplification factor for inflammatory pathways in diabetic vasculature.

Importantly, the role of RAGE in other models of diabetic complications has also been examined. Impaired wound healing often accompanies long-standing diabetes [53–55]. Indeed, slowed or lack of wound healing in diabetic human subjects often results in significant morbidity, such as amputation and, sometimes, mortality. Therapeutic intervention has met with only limited success. Local application of growth factors, such as PDGF-BB, to wounded tissue has not achieved universal improvement in clinical trials [56–58]. To test the hypothesis that

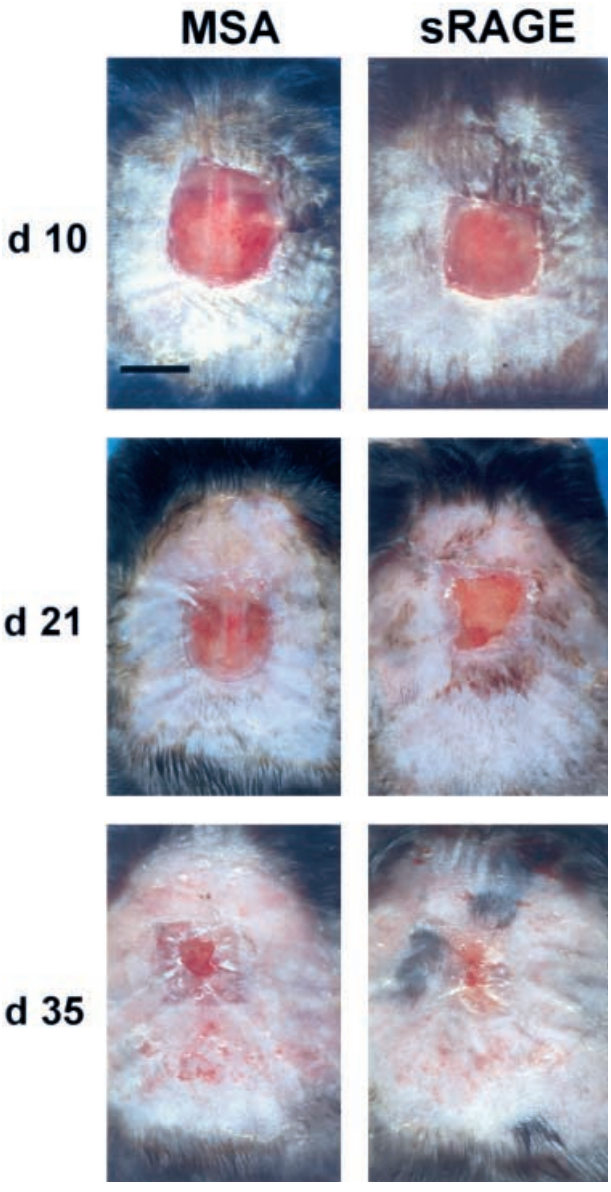

Figure 2. Administration of soluble RAGE accelerates wound healing in db+/db+ mice. Full-thickness excisional wounds (1.5 × 1.5 cm) were created on the backs of male genetically diabetic db+/db+ mice, age 10 weeks. On days 3–10 after wounding, murine sRAGE or MSA was administered topically. Representative wounds from the indicated mice are shown. Scale bar, 1.2 cm.

administration of sRAGE might impact on the biology of wound healing in diabetes, we employed the db/db mouse, in which delayed wound healing occurs after creation of a full-thickness excisional wound. sRAGE, administered either topically or by intraperitoneal administration, caused dose-dependent acceleration of wound healing in this model (fig. 2) [41]. Wound healing consists of established phases: an initial inflammatory phase is followed by proliferative and finally remodelling phases. Our findings suggested that although the initial inflammatory phase was delayed in diabetic mice, once established, inflammation persisted, thereby leading to sustained expression of cytokines and tissue-degradative mediators [41]. Blockade of RAGE, using sRAGE, resulted in acceleration of the inflammatory phase of wound healing. However, once set in motion, blocking access to RAGE limited the inflammatory response, thereby leading to accelerated wound closure [41].

In these wound-healing studies, mice that demonstrated infected wounds were excluded from further study [41]. An important question arising from these experiments was whether blockade of RAGE might impact adversely on host responses to bacterial infection. To address this, we examined a murine model of infection-initiated periodontal disease, a process well-established to be exaggerated in human subjects with diabetes [59–61]. Human and murine infected gingival tissue are enriched in AGEs and display enhanced expression of RAGE, specifically in inflammatory cells such as MPs, in EC, and in cells of the surrounding connective tissue, such as fibroblasts [38]. To address the potential role of RAGE in the pathogenesis of accelerated alveolar bone loss in diabetes, a model system was needed: C57BL/6 mice were rendered diabetic with stz. After 1 month of established diabetes, mice were subjected to oral/anal gavage with the human periodontal pathogen *Porphyromonas gingivalis*. The mice were followed serially for loss of alveolar bone compared with age-matched controls not rendered diabetic. At 2 months after infection, diabetic mice displayed increased loss of alveolar bone compared with nondiabetic mice of the same age [62]. Thus, having established a model of accelerated periodontal bone loss in diabetes, we tested the role of RAGE in these processes. Soluble RAGE was administered immediately upon inoculation with *P. gingivalis*. A dose-dependent suppression of alveolar bone loss was observed in sRAGE-treated mice compared with those receiving MSA [63]. In parallel with decreased bone loss, sRAGE-treated mice displayed decreased gingival levels of AGEs, RAGE, TNF-α, interleukin (IL)-6, and matrix metalloproteinases (MMPs) 3 and 9 [63]. In addition, zymography revealed that activities of MMPs 2 and 9 were significantly reduced in the presence of sRAGE [63]. Importantly, there was no evidence that infection was uncontained in the animals treated with sRAGE, and in neither group of mice, those receiving sRAGE or vehicle, did animals succumb to bacteria or systemic infection. Thus, blockade of RAGE did not appear to suppress innate host responses necessary to suppress and localize infectious foci. In addition, investigation revealed that levels of blood sugar were unaffected by administration of sRAGE, thereby indicating that AGE-RAGE interaction impacted on gingival inflammation and periodontal disease in diabetes by mechanisms distinct from the effects of hyperglycemia itself.

Taken together, these observations link AGE-RAGE interaction to exaggerated inflammatory and tissue-destructive

responses in diabetes. Studies are ongoing to address the potential role of RAGE in the pathogenesis of diabetic nephropathy and other complications. In the case of diabetic nephropathy, it is interesting that RAGE is expressed to enhanced degrees in diabetic podocytes, in both human and murine glomeruli [40]. Recent observations that administration of sRAGE to db/db mice suppressed functional and structural correlates of diabetic nephropathy may lead to the discovery of novel roles for the podocyte in the pathogenesis of diabetic nephropathy [64].

Alzheimer's disease and the amyloidoses

Aβ accumulates in the brains of human subjects with Alzheimer's disease (AD) and has been ascribed a pathogenic role both inside and outside the cell. In the past, a search for cell surface interaction sites for Aβ in extract of bovine lung identified two bands of ~50 and 30–35 kDa. Amino-terminal sequence analysis revealed that both contained sequences for RAGE, the latter likely the extracellular domain, cleaved from full-length forms after proteolysis [9]. Further studies revealed enhanced expression of RAGE, co-localizing with that of Aβ in human AD brain tissue, in neurons, microglia and vascular elements [9]. Aβ bound RAGE in a dose-dependent manner on plastic dishes and on RAGE-transfected cos cells [9]. Indeed, in cell culture models, incubation of Aβ with RAGE-transfected cos cells resulted in cellular activation, as shown by generation of oxidant stress and activation of NF-κB; these observations were suppressed in the presence of anti-RAGE IgG or sRAGE [9]. In addition, Aβ activated microglia by engagement of RAGE, as indicated by increased microglial migration and generation of TNF-α mRNA and protein [9]. These findings provided a mechanism for *direct* Aβ-induced neuronal toxicity, as well as a means to enhance inflammation within Aβ-enriched central nervous system (CNS) elements, by induction of inflammatory, neurotoxic mediators by activated microglia. Indeed, incubation of Aβ with neuronal cells resulted in increased expression of macrophage-colony stimulating factor (M-CSF) [65]. These findings provided a mechanism for enhanced proinflammatory events in an Aβ-enriched setting such as AD [65]. Experiments underway in transgenic mice overexpressing both neuronal Aβ (mutant amyloid precursor peptide) and RAGE suggest that overexpression of RAGE accelerates mechanisms linked to decreased long-term potentiation (LTP) and impaired behavior.

Recent studies have suggested that RAGE interacts more broadly with β sheet fibrils, and is not solely a receptor for Aβ [66]. Specifically, our findings have demonstrated that RAGE interacts with serum amyloid A. In a murine model of amyloidosis initiated by treatment with amyloid-enhancing factor and silver nitrate, administration of sRAGE, or blocking $F(ab')_2$ fragments of anti-RAGE IgG, resulted in decreased accumulation of amyloid, and decreased indices of cellular stress in the spleen, as indicated by diminished expression of cytokines, chemokines, and nuclear translocation of NF-κB [66].

These findings establish an important role for RAGE as a signal transduction ligand for a broad class of β sheet fibrils. These molecules, possessing implications for AD and a range of amyloidoses, may mediate certain of their pathogenic effects by engagement of RAGE. Future studies will address potential roles for blockade of RAGE in human conditions in which these structures accumulate and exert tissue-damaging effects.

RAGE and amplification of proinflammatory pathways

Upon demonstration of RAGE as receptor for AGEs/CML-modified adducts and Aβ/β sheet fibrils, insight was needed into putative natural functions of the receptor, as AGE/CML and β sheet fibrils largely accumulate and exert their effects in pathophysiologic circumstances. A series of affinity chromatography studies and RAGE-binding experiments led to the identification of two polypeptides that bound RAGE. The first, now identified as S100A12 was called EN-RAGE (Extracellular Newly Identified RAGE-binding protein) [10]. This molecule and related members of the S100/calgranulin family of proinflammatory cytokines [67] are expressed especially in inflammatory cells, particularly within the intracellular milieu. Inside the cell, these molecules are associated with functions such as calcium binding, phagocytosis and cellular motility [67]. However, a view is clearly emerging that these molecules may gain exit to the extracellular environment, thereby allowing them to engage cell surface molecules, such as RAGE [67]. To rigorously test this concept, we examined the properties of these molecules as ligands of RAGE.

We employed a prototypic S100/calgranulin, EN-RAGE. Radioligand-binding studies revealed specific binding of ^{125}I-EN-RAGE to RAGE immobilized on plastic wells, with a K_d ~91 ± 29 nM [10]. Specificity of binding was shown by inhibition in the presence of excess sRAGE, a truncated form of the receptor spanning the extracellular domain, or anti-RAGE IgG. In contrast, an unrelated protein, bovine serum albumin, or nonimmune IgG were without effect. Importantly, another member of this family, S100B, also suppressed ^{125}I-EN-RAGE interaction with RAGE. Human umbilical vein endothelial cells (HUVECs) incubated with EN-RAGE displayed induction of vascular cell adhesion molecule-1 and enhanced binding of VLA-4-bearing Molt-4 cells [10]. Nuclear extracts from EC exposed to EN-RAGE were subjected to electrophoretic mobility shift assay. These findings re-

vealed increased activation of NF-κB in a RAGE-dependent manner, as confirmed by the inhibitory effect of anti-RAGE IgG or sRAGE added to the EC prior to EN-RAGE. EN-RAGE induction of NF-κB nuclear translocation resulted from RAGE-mediated intracellular signalling, as shown by experiments using a truncated form of the receptor, DN-RAGE, from which the cytosolic tail was deleted. EC transfected with DN-RAGE displayed marked suppression of NF-κB activation compared with those transfected with vector alone. Similar results were observed with S100B [10].

As MPs express RAGE and central amplifiers of the inflammatory response, we studied the effects of ligation of MP RAGE by EN-RAGE. Using modified chemotaxis chambers, EN-RAGE placed in the lower compartment stimulated migration of human MPs added to the upper compartment in a dose-dependent manner. In contrast, replacement of EN-RAGE with albumin was without effect on cell migration [10]. These results were inhibited in the presence of sRAGE or anti-RAGE. Incubation of mock-transfected (vector alone) cultured murine MP-like BV2 cells with EN-RAGE caused elaboration of IL-1β and TNF-α into cellular supernatants in a dose-dependent manner. That intact intracellular signalling pathways triggered by RAGE were essential for expression of these cytokines was demonstrated by the striking inhibition observed following transfection of a construct encoding DN-RAGE [10]. In addition, in peripheral blood mononuclear cells which express RAGE, incubation with EN-RAGE resulted in increased proliferation and elaboration of IL-2 [10].

These concepts were addressed in vivo employing two models of inflammation. In the first, a murine model of delayed-type hypersensitivity was employed. Mice were sensitized and challenged with methylated bovine serum albumin (mBSA; not a ligand for RAGE) [68]. The contribution of EN-RAGE interaction with RAGE to the pathogenesis of inflammation in this model was tested by administration of sRAGE or blocking $F(ab')_2$ fragments of anti-RAGE/anti-EN-RAGE IgG [10]. Administration of murine sRAGE or anti-RAGE/anti-EN-RAGE $F(ab')_2$ fragments by intraperitoneal injection resulted in dose-dependent suppression of inflammation in mBSA-sensitized/challenged mice; at an sRAGE concentration of 100 μg/dose, the inflammation score was reduced to virtually the background levels observed in mice without sensitization/challenge [10]. Consistent with these results, hematoxylin and eosin staining revealed striking abrogation of inflammatory cell influx and formation of granulomata, and decreased footpad edema in animals treated with sRAGE [10]. In parallel with decreased indices of inflammation, further examination revealed decreased mRNA and protein levels for IL-2 and TNF-α in extracts prepared from the footpads subjected to blockade of RAGE. In addition, activation of NF-κB was significantly suppressed in the nuclear extracts prepared from footpads retrieved from mice treated with sRAGE [10].

To address the role of S100/calgranulin-RAGE interaction in a model of chronic inflammation, we studied IL-10 null mice in which colonic inflammation develops in parallel with increasing weight loss and anemia [69]. Interestingly, a hallmark of S100/calgranulin polypeptides is their association with chronic inflammation, such as human inflammatory bowel diseases [70, 71]. Beginning at age 4 weeks, IL-10 null mice were treated with either MSA or sRAGE for 6 weeks. At sacrifice, sRAGE-treated mice displayed decreased inflammatory infiltrates in the colonic tissue. When nuclear extracts were prepared from the colonic tissue, significantly decreased nuclear translocation of NF-κB was observed in mice treated with sRAGE [10].

Recent studies have extended these concepts to other models of inflammation. For example, pilot experiments in murine models of experimental autoimmune encephalitis induced by sensitization/challenge with myelin basic protein and bovine type II collagen-induced arthritis strongly suggest that blockade of RAGE may be a propagation factor in inflammatory diseases. Importantly, in both of these settings, blockade of RAGE did not inhibit generation of antibodies to the respective antigens. Rather, driven by increased accumulation of S100/calgranulins borne largely by invading inflammatory cells, activation of RAGE appears to be an integral component of host responses that sustain the inflammatory response (fig. 3). Thus, by cutting off the proinflammatory cascades perpetuated, at least in part via RAGE, resolution of inflammation ensues, processes that yield tissue repair rather than irreparable damage.

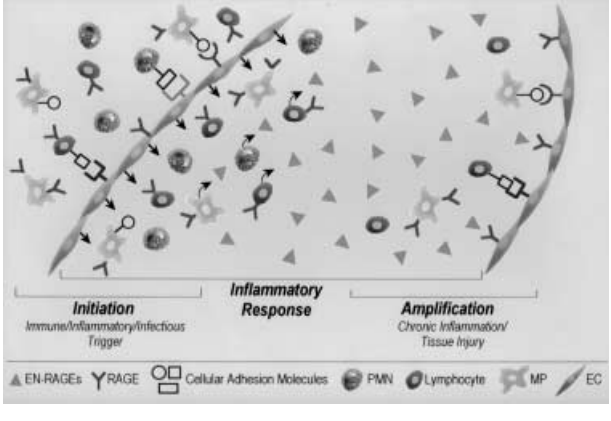

Figure 3. S100/calgranulin and RAGE interaction: an amplification pathway in immune/inflammatory disorders. We hypothesize that S100/calgranulin-RAGE interaction is not a cause of immune/inflammatory disorders. Rather, we speculate that inflammatory cells, once attracted to sites of immune/inflamed foci release S100/calgranulins, thereby providing a mechanism for these molecules to engage RAGE and perpetuate cellular activation and increase the likelihood of chronic disease.

RAGE, cellular motility and tumor biology

Development

At the time we identified S100/calgranulins as ligands for RAGE, column chromatography and radioligand-binding experiments yielded an additional polypeptide capable of binding RAGE. Based on amino-terminal sequence analysis, this polypeptide was shown to be amphoterin [11]. Interestingly, the biology of amphoterin appears to be relevant in both homeostasis and disease. Amphoterin was first described in the setting of neuronal development [72, 73]. Rauvala and colleagues were the first to show that amphoterin mediated neurite outgrowth in neurons retrieved from embryonic rats (day 17). We extended these observations to show that RAGE was expressed to enhanced degrees in developing neurons of the CNS; for example, cerebral, cerebellar and hippocampal neurons demonstrated RAGE mRNA and antigen [11]. In these studies, RAGE expression overlapped with that of amphoterin. Studies on the promoter of RAGE suggested that Sp1-binding elements might mediate the effects of amphoterin on up-regulating neuronal expression of RAGE [74]. To determine the potential role of RAGE in mediating neurite outgrowth by this receptor, we performed several studies.

To confirm that amphoterin bound RAGE, radioligand-binding assays were performed; amphoterin purified from rat brain or recombinant material bound purified sRAGE in a dose-dependent manner, with K_d ~6.4 ± 1.0 nM [11]. Similarly, amphoterin bound RAGE-bearing cultured neurons excised from rat embryos in a manner dependent on RAGE [11]. The key test of the hypothesis that RAGE mediated the effects of amphoterin in neurite outgrowth was shown by the selective inhibition of neurite outgrowth by sRAGE or anti-RAGE IgG on amphoterin-coated matrices, not on matrices such as poly-L-lysine (fig. 4) [11]. These experiments demonstrated that the effects of amphoterin on neurite outgrowth were likely receptor mediated, and that RAGE, in particular, was a key cell surface receptor for this molecule.

These observations suggested that perhaps attempts at generation of RAGE null mice might be futile. However, recently, a RAGE null mouse has been developed [11]. Preliminary studies indicate that in the absence of RAGE, mice are viable, with normal reproductive fitness. We speculate that uncovering the phenotype of these mice will best be done in the settings of stress. Thus far, we have shown that RAGE null mice display markedly reduced neointimal expansion after femoral artery injury, a finding that appears to be importantly related to the effects of ligand-RAGE engagement in VSMCs [11].

Tumor biology

In addition to increased expression of amphoterin in neuronal development, Rauvala and colleagues demonstrated that amphoterin was highly expressed in migrating cells, such as tumors [75]. This observation prompted the hypothesis that RAGE might be involved in tumor cell migration. To study this, we began with murine models of tumors raised from injected tumor cells. Rat C6 glioma cells were an ideal starting point for these studies, as immunoblotting of C6 glioma cell lysates demonstrated expression of both RAGE and amphoterin. Administration of sRAGE once daily to immunocompromised (athymic nude) mice upon injection of rat C6 glioma cells caused dose-dependent decreases in tumor volume [76]. Furthermore, administration of monospecific polyclonal rabbit $F(ab')_2$ fragments prepared from antibodies to RAGE or amphoterin to immunocompromised (severe combined immunodeficiency, SCID) mice from the time of inoculation with C6 glioma cells revealed significantly reduced tumor volume in the presence of either anti-amphoterin or anti-RAGE $F(ab')_2$ after 21 days [76]. However, rabbit nonimmune $F(ab')_2$ fragments were without effect [76].

To be certain that the effects observed on tumor reduction were specifically due to RAGE, we prepared clones by limiting dilution of stably transfected C6 glioma cells as follows: mock-transfected C6 glioma cells (M); C6 glioma expressing full-length RAGE (F), C6 glioma ex-

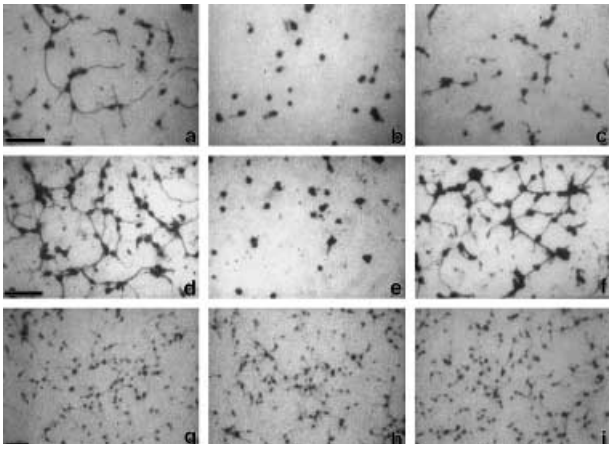

Figure 4. Blockade of RAGE suppresses neurite outgrowth induced by amphoterin. Eight-chamber wells were coated with either amphoterin (*a–f*) or poly-L-lysine (*g–i*) for 18 h. Cortical neuronal cells were isolated from E17 rat embryos and fixed with paraformaldehyde (4%) containing NP-40 (0.1%) and stained with monoclonal anti-tubulin antibody. Scale bars, 50 µm. (*a–c*) Effect of sRAGE. Amphoterin-coated wells and neuronal cells were pretreated with sRAGE or bovine serum albumin for 1 h at 37 °C: neurite outgrowth on amphoterin-coated wells alone (*a*), in the presence of sRAGE (50 µg/ml) (*b*) or in the presence of sRAGE (5 µg/ml) (*c*). (*d–f*) Neurite outgrowth in the presence of anti-RAGE $F(ab')_2$ or nonimmune $F(ab')_2$: in the presence of nonimmune $F(ab')_2$ (40 µg/ml) (*d*) or an ti-RAGE $F(ab')_2$ 40 µg/ml) (*e*) or 4 µg/ml (*f*). (*g–i*) Neurite outgrowth on poly-L-lysine coated wells and the effect of RAGE blockade: neurite outgrowth on poly-L-lysine alone without inhibitor (*g*), in the presence of sRAGE (50 µg/ml) (*h*) or anti-RAGE $F(ab')_2$ (40 µg/ml) (*i*).

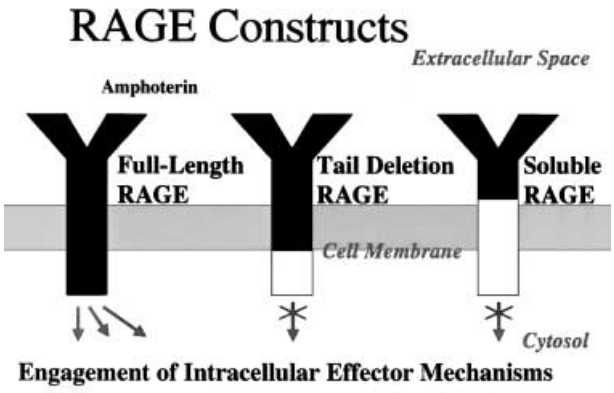

Figure 5. Preparation of RAGE-modified constructs. Constructs were prepared to study the role of RAGE and RAGE signalling in tumor biology. In addition to empty vector, constructs were prepared to overexpress full-length functional RAGE, tail-deletion RAGE (imparts a dominant negative effect) and soluble RAGE (to produce decoy that binds up ligand and prevents activation of cell surface RAGE).

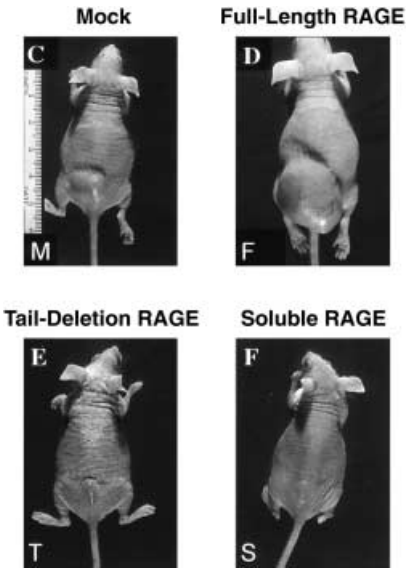

Figure 6. RAGE blockade suppresses growth of implanted C6 glioma. Mock or RAGE/RAGE mutant C6 glioma cells were injected into immunocompromised mice. Representative photographs on day 21 of mice bearing the indicated transfected clone are shown.

pressing soluble RAGE (S), and C6 glioma expressing tail-deletion/DN-RAGE (T) (fig. 5). When implanted in immunocompromised mice, compared to mock-transfected tumors, those bearing full-length RAGE were significantly larger and more invasive [76]; those bearing sRAGE or tail-deleted RAGE were strikingly smaller (fig. 6) and less invasive [76].

In parallel with decreased tumor volume and invasiveness, levels of active MMPs were reduced in sRAGE- or tail-deletion RAGE-expressing tumors [76]. New insights into mechanisms by which activation of RAGE might contribute to increased tumor size, however, emerged upon examination of tumor proliferation. Compared to mock transfectants, C6 glioma expressing full-length RAGE exhibited enhanced incorporation of 5-bromo-2′-deoxyuridine (BrdU) on days 1 and 3 after implantation. In contrast, tail-deletion RAGE and sRAGE transfectants exhibited a decrease in incorporation of BrdU on days 1, 3 and 7 compared with mock-transfected clones. However, rates of apoptosis were low (0.2–0.5% from days 1–14) and did not differ among the various transfectants. Studies are ongoing to examine the mechanisms by which ligand engagement of RAGE results in increased tumor cell proliferation.

The key test of these concepts was whether RAGE contributed to tumor metastases, as both tumor growth and invasion are critically linked to the ability of tumors to spread from their local milieu. We employed the Lewis lung carcinoma model, in which lung metastases rapidly develop upon removal of primary tumor. Both RAGE and amphoterin were present in Lewis lung carcinoma cells [76]. Soluble RAGE was administered just prior to and after resection of primary tumors resulting from inoculation with wild-type Lewis lung carcinoma cells. Compared with vehicle, animals receiving the highest dose of sRAGE demonstrated a marked decrease in the number of lung surface metastases (8.7 ± 1.4 vs 1.0 ± 0.3; $p < 0.0001$) and metastatic burden as judged by lung weight (385.6 ± 39.8 vs 188.7 ± 6.7 mg; $p < 0.001$) (fig. 7). Lung surface metastases observed in MSA-treated mice were virtually undetectable in mice treated with sRAGE (fig. 7) [76].

Importantly, in addition to tumors raised from implant cells, blockade of RAGE also suppressed tumor growth in endogenously forming papilloma driven by expression of the v-Ha-*ras* transgene [76]. In this model, and in a murine model of breast cancer driven by the murine mammary tumor virus promoter, blockade of RAGE using sRAGE suppresses local tumor growth and distant metastases.

In our first studies, blockade of RAGE did not appear to affect neoangiogenesis within the tumors [76]. Further studies are in progress to explore in greater depth the possibility that RAGE may impact on this key process within the tumor bed.

RAGE and intracellular signalling

Our experiments employing tail-deletion RAGE strongly suggested that ligand binding to the receptor exerted cellular effects by engagement and activation of intracellular signalling molecules. Studies from our laboratory and others suggested that multiple signalling molecules may be recruited by activation of RAGE, such as $p21^{ras}$, p44/p42 MAP kinase, p38 and SAPK/JN kinases, NF-

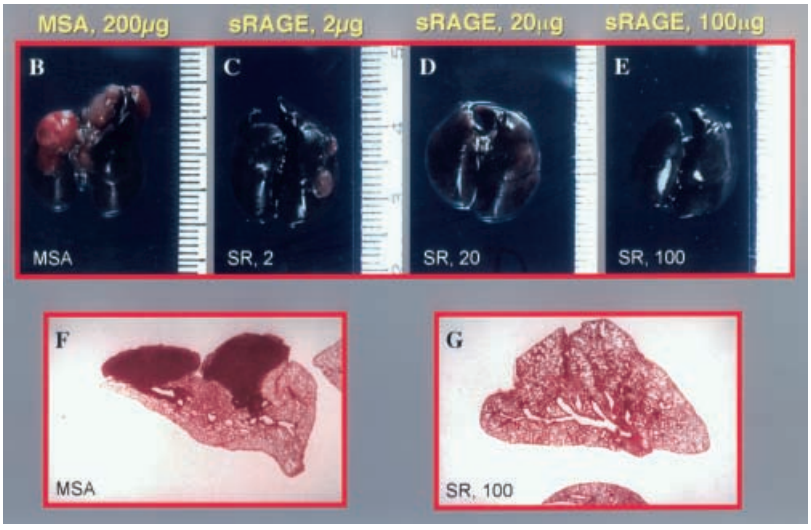

Figure 7. RAGE blockade suppresses tumor metastases. Lung surface metastases were reduced in a dose-dependent manner by treatment with sRAGE versus vehicle MSA. (The tracheae were infused with India ink prior to photography). Representative histology of lung metastases is shown in MSA-treated mice (*F*) vs sRAGE, 100 μg/day (*G*).

κB, cdc42/rac and the JAK/STAT pathways [76–82]. We speculate that depending on the ligand and, perhaps, on the state of differentiation of RAGE-bearing cells, distinct pathways may be activated, thereby determining the phenotype and outcome in the cellular environment. Indeed, in certain settings, activation of RAGE appears to modulate behavior of molecules linked to the inflammatory response; sustained and chronic activation of NF-κB may emerge upon activation of RAGE by its ligands [82]. Thus, in addition to sustained expression of RAGE in ligand-enriched milieu, consequences of this interaction in terms of cellular activation may be perpetuated.

In addition to dissecting the full range of signalling molecules impacted upon by RAGE, a critical challenge will be to identify the immediate intracellular molecules recruited by the cytosolic domain of RAGE upon activation of the receptor. In this way, we speculate that it will be possible to uncover the *natural* functions of the molecule, as well as the means by which activation of the receptor in pathophysiologic states leads to chronic cellular activation.

RAGE: implications for homeostatic functions

Insights into putative natural functions of RAGE have emerged from considering the concept that limited inflammatory responses may be beneficial to certain stresses in the host. Especially in settings where ligands of the receptor are present, short-lived inflammation may serve to foster repair and remodelling. Specifically, after crush of the sciatic nerve, expression of both RAGE and its ligands, amphoterin and S100/calgranulins, is increased temporarily; by 21 days after injury, expression of these molecules returns to baseline. Thus, in contrast to chronic disease, in which sustained expression of ligands and RAGE appear not to be down-regulated, the situation in peripheral nerve appears quite distinct. To study potential reparative roles for RAGE after nerve injury, mice were subjected to unilateral sciatic nerve crush in the presence of sRAGE, blocking antibodies to the receptor/ligands or vehicle. Blockade of RAGE impaired Wallerian degeneration and removal of myelin A critical consequence of ineffective Wallerian degeneration in this model was the failure to optimally regenerate and myelinate new nerve fibers [83]. Thus, in addition to highlighting physiologic roles for RAGE-mediated inflammation, these studies suggest that neurite outgrowth via RAGE may be essential for realizing the full regenerative phenotype after nerve crush [83]. Indeed, one view of peripheral nerve regeneration after injury casts the picture of regeneration as a recapitulation of developmental processes [83]. From this point of view, the biology of RAGE may be relevant in both development and response to cellular stresses.

Conclusion

Our recent studies have focussed largely on the biology of RAGE from the ‘outside’. Ligand enrichment within the extracellular milieu appears to importantly regulate expression and activity of the receptor. In this context, ligand-induced activation of RAGE is likely to be involved in the pathogenesis of chronic tissue injury associated with settings of ligand accumulation. Although seem-

ingly a diverse group of disorders, the classes of ligands interacting with RAGE are the unifying element which associate the receptor with amplification of host responses in disorders such as diabetes, inflammation, neurodegeneration and tumors. Thus, this work leads to the question of whether blockade of RAGE in human conditions is a feasible strategy for prevention/stabilization of chronic diseases. Where do we go from here? Clearly, the next step is to rigorously focus on the effects of RAGE activation 'inside' the cell. Work is ongoing to uncover the site and time dependence of engagement of intracellular signalling molecules and their relevant adaptor molecules triggered by activation of RAGE. Only by thorough dissection of the biology of RAGE 'inside' and 'outside' the cell will it be possible to identify the efficacy and safety of strategies designed to block the receptor in human disease.

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