Review

Arguing for the motion: Yes, RAGE is a receptor for advanced glycation endproducts

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Advanced glycation endproducts (AGEs) are an heterogenous class of compounds formed by diverse stimuli, including hyperglycemia, oxidative stress, inflammation, renal failure, and innate aging. Recent evidence suggests that dietary sources of AGE may contribute to pathology. AGEs impart diverse effects in cells; evidence strongly suggests that crosslinking of proteins by AGEs may irrevocably alter basement membrane integrity and function. In addition, the ability of AGEs to bind to cells and activate signal transduction, thereby affecting broad properties in the cellular milieu, indicates that AGEs are not innocent bystanders in the diseases of AGEing. Here, we present evidence that receptor for AGE (RAGE) is a receptor for AGEs.

Keywords: Advanced glycation endproduct / Glycation / RAGE / Receptor / Signal transduction

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This article focuses on pro arguments about "Dietary AGEs and ALEs interact with RAGE".
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1 Introduction

The receptor for advanced glycation endproducts (RAGE) is a multiligand receptor of the Ig superfamily. Although RAGE was first described as a signal transduction receptor for the products of nonenzymatic glycation and oxidation of proteins and lipids, the advanced glycation endproducts, or AGEs [1, 2], it is clear that the biology of this molecule is more complex. Among the ligand families of RAGE are S100/calgranulins; high mobility group box1 (HMGB1), also known as amphoterin; amyloid-β peptide (Aβ) and β-sheet fibrils; and Mac-1 [3 – 8].

In this review, we focus on the *in vitro* and *in vivo* evidence supporting the premise that RAGE is a receptor for AGEs.

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Abbreviations: AGE, advanced glycation endproduct; **CML,** carboxymethyl lysine; **EC,** endothelial cells

2 The discovery of RAGE: Identification of an AGE-binding protein in bovine lung extract

Studies suggesting that AGEs prepared *in vitro* by the incubation of native proteins with high concentrations of glucose, glucose-6-phosphate, or ribose bound in a dose-dependent and saturable manner to cultured endothelial cells (EC) stimulated the hypothesis that EC expressed cell surface receptors for AGEs [1]. Bovine lung powder, highly enriched in microvascular EC, was subjected to extraction with the detergent octyl- β-glucoside and probed for binding activity to ^{125}I-AGE-BSA. Through a series of multiple chromatographic steps, RAGE was ultimately purified to homogeneity. AGE bound bovine RAGE purified from the lung extract when placed on plastic wells. The binding of AGE to RAGE displayed binding affinity in the range of $K_d \approx 50$ nM [1]. Multiple studies subsequent to those observations demonstrated that binding was inhibited in the presence of antibodies raised to bovine RAGE; human RAGE was subsequently cloned from a lung library and shown to bind AGE as well [2].

Subsequent experiments revealed that distinct classes of reducing sugars used to prepare AGEs raised species that bound RAGE; thus, whether prepared from protein incubated in glucose, glucose-6-phospate or ribose, the resulting AGE material, but not the backbone protein, bound

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RAGE [9]. At the same time, experiments revealed that in addition to EC, distinct cell types bound AGE, such as mononuclear phagocytes [9].

A clear caveat of *in vitro*-prepared AGE is that these species are: (i) highly heterogenous; AGE constitutes many different forms of stable endproducts; (ii) the biologically relevant degrees of modification in AGE prepared *in vitro* that mimic *in vivo*-generated AGE are not well-known; and (iii) the biologically relevant concentrations of *in vitro*-prepared AGE are not well established. In this context, the relevant species in the circulation *versus* those in the vessel wall in diabetes or renal failure are not readily definable, especially as some may be highly crosslinked and relatively insoluble. Thus, a critical step in AGE–RAGE studies was made when, in addition to strictly *in vitro*-prepared AGEs, AGEs affinity-purified and prepared from human subjects with diabetes, using anti-AGE IgG as the bait, were found to activate cultured EC, *via* RAGE, and upregulate vascular cell adhesion molecule-1 (VCAM-1) [10]. These findings ensured that material retrieved directly from AGE-enriched subjects bore potential to bind and activate RAGE and downstream consequences.

3 Specific AGE that bind RAGE: Carboxymethyl lysine (CML) AGE are RAGE ligands

In addition to heterogenous AGEs, it was important to delineate specific AGEs capable of binding RAGE. Toward this end, CML AGEs were selected and tested for their ability to bind RAGE. CML-AGE accumulated in hyperglycemia, oxidative stress, and inflammation – biologically relevant milieu in the context of RAGE – and thus were a logical specific AGE to test. CML-AGE was prepared and characterized and shown to bind RAGE both in radioligand binding assays and on cultured cells in a manner similar to that observed with heterogenous AGE species [11].

CML-AGE-RAGE binding and activation of EC, macrophages and smooth muscle cells (SMC) was blocked by preincubation of the cells with anti-RAGE IgG or soluble RAGE, the extracellular ligand binding domain of RAGE, or by introduction of a construct in which the cytoplasmic domain of the receptor was deleted, thereby imparting a dominant negative or DN effect. These data linked CML-AGE to RAGE *via* signal transduction; indeed, one of the targets of CML-RAGE interaction was activation of the key factor, NF-kB [11]. In other studies, CML-AGE bound RAGE-expressing mesothelial cells, resulting in release of vascular endothelial growth factor (VEGF) and potentiation of capillary tube formation [12].

In vivo, infusion of CML-BSA to normal mice resulted in upregulation of VCAM-1 in the lung; a process prevented by pretreatment of the mice with either anti-RAGE IgG or sRAGE, but not by nonimmune IgG [11]. These data provided the first evidence that CML-AGE bound and activated RAGE, both *in vitro* and *in vivo*.

4 RAGE and the expanding repertoire of its AGE ligands

It is not surprising nor unexpected given the diverse settings in which AGEs may form, that highly varied AGE species may be identified. In addition to CML-AGE molecules, the recent identification that RAGE-expressing HEK 293 cells bound pronyl glycine, a compound formed in association with the process-induced heat impact applied to bread dough, and activated mitogen activated protein (MAP) kinases, expanded the metabolic context of RAGE to dietary AGE biology [13]. Further, the observation that RAGE bound methylglyoxal-modified albumin (MGO-albumin) in macrophages provided new insights into means by which products of highly reactive dicarbonyls might interact with RAGE and activate signaling pathways [14].

5 *In vivo* studies – role of blocking RAGE in AGE-enriched environments

To test the role of AGE–RAGE interaction *in vivo*, we and others examined murine models of diabetic complications in which distinct interventions might be employed to study and dissect the role of RAGE. The role of RAGE was explored in diabetes-associated nephropathy. A murine model of type 2 diabetes was employed to test the role of RAGE; in vehicle-treated db/db mice, a sustained but nonprogressive increase in urinary albumin excretion was observed, in parallel with increased expansion of the mesangial matrix. When sRAGE was administered to these animals from 8 wk and continued until approximately 6 months of age, a significant reduction in albumin excretion and mesangial matrix was observed [15]. When antibodies to RAGE were administered chronically to diabetic mice, markers of nephropathic changes were reduced [16]. Furthermore, in the AGE-enriched environment of diabetes, overexpression of functional RAGE in vascular cells enhanced nephropathy, in a manner prevented by administration of AGE inhibitors [17]. Additionally, homozygous deletion of RAGE resulted in significant protection against nephropathy-linked pathology and albumin excretion [15, 18]. Although it is not possible to fully exclude the impact of distinct RAGE ligands in this setting, it is nevertheless clear that the impact of AGE was attenuated in RAGE null mice. It is important to note that anti-AGE strategies also imparted benefit in rodent models of diabetic nephropathy, thus, clarifying that indeed AGEs do contribute to the pathogenesis of kidney disease in diabetes in these models [19, 20].

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In murine models of diabetic neuropathy induced by streptozotocin, administration of sRAGE significantly reduced neuropathic changes consistent with long-term diabetes. Similar levels of protection were observed in RAGE null mice, suggesting that deletion of RAGE abrogated the aberrant effects of AGE [21].

Studies in atherosclerosis-prone mice deficient in apolipoprotein E revealed that treatment with sRAGE prevented the early acceleration of atherosclerosis in streptozotocin-treated mice, and in mice with established diabetic atherosclerosis, stabilized atherosclerosis in these animals [22, 23]. When plasma retrieved from sRAGE-treated animals was subjected to immunoprecipitation with anti-RAGE IgG, bound material was immunoblotted with anti-AGE IgG, suggesting that circulating AGEs were bound to RAGE and, therefore, that administration of sRAGE bound AGEs and blocked their interaction with and activation of the cell surface receptor.

Experiments have elucidated that AGEs may form even in nondiabetic environments. We tested these concepts in a model of ischemia/reperfusion injury in the isolated perfused heart. Administration of sRAGE to mice or rats prior to I/R in the isolated perfused heart revealed significant protection against necrosis and functional deficits. In an analogous manner, homozygous RAGE null mice hearts displayed significant protection against injury in this model. These data led us to consider that I/R itself may generate RAGE ligands. Indeed, consistent with this premise, we observed that AGEs were produced in the I/R environment and that their production was reduced in RAGE null mice [24]. Taken together, in diabetic and nondiabetic environments, a unifying feature of injury in these settings appears to be the generation of AGE. AGE interaction with RAGE is a principal mechanism by which AGEs impart injury; blockade of RAGE, with either the ligand-binding decoy sRAGE, antibodies to RAGE or genetic deletion of RAGE (RAGE null mice) protected the organism from the adverse impact of AGE ligands.

6 AGE–RAGE in human subjects; tracking sRAGE may provide a novel biomarker for AGE burden and disease

An emerging body of evidence suggests that circulating levels of sRAGE may serve, at least, as biomarkers for the degree of injury in organisms affected by chronic diseases. For example, a body of evidence is emerging indicating that lower levels of sRAGE appear to reflect enhanced vulnerability to diabetes complications, coronary artery disease, Alzheimer's disease, and inflammatory arthritis [25–28]. It is possible that the distinct levels of sRAGE in health and disease reflect ligand burden. Alternatively, or in addition, it is possible that levels of sRAGE are, at least in part, genetically primed, thereby highlighting the possibility that the ability to bind up RAGE ligands by increased levels of sRAGE may be genetically controlled. Evidence from human studies suggests that ligand binding may, indeed, be one mechanism regulating levels of sRAGE. Indeed, in one study, aged centenarians were found to have very high levels of sRAGE, even higher than healthy young subjects and especially young subjects with cardiac disease [29].

Levels of sRAGE may be modulated in human subjects consequent to therapeutic interventions. Human subjects with type 1 diabetes treated with the angiotensin converting enzyme inhibitor (ACEi) perindopril displayed higher levels of sRAGE consequent to therapy, and, most importantly, complexes between sRAGE and the specific AGE CML were identified in human plasma [30]. Similar observations were noted *in vivo* in diabetic rats treated with the ACEi ramiprilat [30]. These data, similar to studies in sRAGE-treated diabetic mice with atherosclerosis, provide further evidence that AGEs bind RAGE.

7 RAGE is a signal transduction receptor for AGEs

It is important to note that beyond RAGE, distinct receptors may bind AGEs, such as macrophage scavenger receptor (MSR) type II, OST-48, 80K-H, galectin-3, CD36 [31–34]. Published data suggest that these distinct receptors may not play significant roles in mediating signal transduction *via* RAGE. Rather, unlike RAGE, they may serve roles more relevant in AGE detoxification. As discussed above, introduction of signal transduction deficient mutants of RAGE into cultured cells fully abrogates the impact of CML-AGE. Indeed, in *in vivo* studies, transgenic mice expressing signal transduction deficient mutants of RAGE also fail to modulate gene expression and alter organism phenotype, at least in part *via* failure to activate NF-kB [35, 36].

Previous studies revealed that in nondiabetic mice subjected to acute femoral artery endothelial denudation, rapid formation of CML AGEs was noted in the injured femoral artery. Our preliminary data confirm this result and indicate key pre-AGE species, such as MGO, are also rapidly generated consequent to arterial injury. Suggestive of key roles for AGE–RAGE interaction in mediating enhanced neointimal expansion, administration of sRAGE or blocking antibodies to RAGE *in vivo* reduced neointimal expansion. Further, in homozygous RAGE null mice, neointimal formation was greatly suppressed. Indicative of definitive roles for AGE–RAGE signaling in neointimal expansion, signal transduction mutant mice (in SMC driven by the SM22 alpha promoter) displayed significantly reduced intima–media (*I*/*M*) ratio. Although it is possible that distinct ligands of RAGE contributed to neointimal expansion, the enhanced production of AGE directly in the injured vessel wall clearly implicates RAGE as a chief receptor for injury-provoking species [36].

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8 Concluding comments

The fascinating nature of RAGE is its ability to bind both AGE and non-AGE ligands – all linked in key ways to inflammatory mechanisms. Ongoing work suggests that these ligands share more similarities than differences. For example, the identified ligands of RAGE may exist in oligomeric forms and, when in the oligomeric form, appear to display enhanced ability to activate RAGE [37]. Thus, we propose that such forms are the key interfaces linking these seemingly diverse ligands directly to RAGE. Indeed, recent studies suggest that glycation may promote amyloid formation [38].

Recent studies are providing novel insights into the precise structural determinants underlying these findings, including the observations that RAGE ligands crosscompete in binding assays (AGE, S100, and HMBG1) and bind to the V-domain of RAGE [3, 11]; that HMGB1 (amphoterin) binding to RAGE is partially dependent on glycosylation (N-glycans) of the V-type Ig domain [39]; and that S100A12, particularly in hexameric form, may bind to the C-type Ig domain of RAGE [40]. In conclusion, we predict that further studies dissecting the physical mechanisms by which ligands bind RAGE may yield critical insights toward identification of multiple classes of RAGE antagonists.

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