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Receptor for AGE (RAGE) & its Ligands – Cast into Leading Roles in Diabetes & the Inflammatory Response

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Abstract

The actors in the pathogenesis of diabetes and its complications are many and multi-faceted. The effects of elevated levels of glucose are myriad; among these are the generation of Advanced Glycation Endproducts (AGEs), the products of nonenzymatic glycoxidation of proteins and lipids. The finding that AGEs stimulate signal transduction cascades through the multi-ligand receptor RAGE unveiled novel insights into diabetes and its complications. Inextricably woven into AGE-RAGE interactions in diabetes is the engagement of the innate and adaptive immune responses. Although glucose may be the triggering stimulus to draw RAGE into diabetes pathology, consequent cellular stress results in release of pro-inflammatory RAGE ligands S100/calgranulins and HMGB1. We predict that once RAGE is engaged in the diabetic tissue, a vicious cycle of ligand-RAGE perturbation ensues, leading to chronic tissue injury and suppression of repair mechanisms. Targeting RAGE may be a beneficial strategy in diabetes, its complications, and untoward inflammatory responses.

Keywords

receptor; glycation; diabetes; complications

The Receptor for Advanced Glycation Endproducts (RAGE) was first identified as an endothelial cell (EC) surface receptor for the products of nonenzymatic glycation and oxidation of proteins and lipids, the Advanced Glycation Endproducts (AGEs) [1]. This finding linked RAGE to hyperglycemia and to distinct settings characterized by accelerated production and accumulation of AGEs, such as aging, renal failure, and inflammation [2]. Since the identification of RAGE, expansion of these concepts has occurred on a number of fundamental fronts. First, RAGE biology is not limited to that of the AGEs. RAGE is a multi-ligand receptor whose interaction with certain members of the S100/calgranulin family (such as S100A12 and S100B), High Mobility Group Box-1 (HMGB1), amyloid- β peptide (A β) and β -sheet fibrils, and Mac-1 implicates the receptor in disorders in which these ligands accumulate, such as inflammation, cancer, neurodegeneration, and amyloidoses [3–7]. Second, beyond EC, RAGE is expressed on multiple other cell types, such as smooth muscle cells (SMC), monocytes/macrophages, T lymphocytes, dendritic cells (DC), glomerular epithelial cells or podocytes, cardiomyocytes, neurons of the central and peripheral nervous systems and transformed cells [8]. Third, RAGE is a signal transduction receptor. Multiple studies have illustrated that depending on the cell type and the stimulus, a variety of signal transduction cascades may be activated. That signal transduction is essential for the biological actions of RAGE has been illustrated by

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experiments, both *in vitro* and *in vivo*, in which RAGE ligand-dependent actions are blocked upon deletion of the cytoplasmic domain of RAGE [3,5,9]. In cells or in transgenic mice stimulated in a RAGE ligand environment, deletion of the RAGE cytoplasmic domain imparts a dominant negative (DN) effect. Finally, compelling experiments using pharmacological antagonists of ligand/RAGE or RAGE genetically-modified mice have suggested that this receptor plays key roles in diabetes and its complications and inflammation, as well as neurodegeneration and cancer.

In this review, we will discuss the state of RAGE with a particular focus on its roles in diabetes, and how this biology is immersed in the immune response.

The Ligands of RAGE – setting the stage for diabetes and its complications & beyond

AGEs

AGEs are a heterogeneous group of structures in which nonenzymatic glycation on lysine and arginine residues results in fundamental alterations in protein signature [10]. Once glycated, AGE epitopes on proteins and lipids may gain new functions via interaction with their chief signal transduction receptor, RAGE [1,11]. As AGEs are quite heterogeneous, it is likely that not all interact with RAGE. However, a common AGE that accumulates in diabetes, carboxy methyl lysine (CML) AGE, binds RAGE and modulates cellular properties [9]. AGEs accumulate in diabetic tissues. Interestingly, in diabetic kidney and heart without superimposed injuries, homozygous RAGE null mice display lower levels of AGEs versus those measured in RAGE-expressing animals. In the heart, diabetic RAGE null mice (C57BL/6 background) displayed lower levels of CML, pentosidine and furosine AGEs compared to wild-type mice [12]. As levels of glycemia were not different among these groups of diabetic animals, these data suggest that factors accelerating AGE formation in the hyperglycemic environment were modulated by RAGE. One such factor, oxidative stress, contributes to AGE generation. *In vitro* and *in vivo*, activation of RAGE activates NADPH oxidase and generates reactive oxygen species (ROS) [13]. Thus, a revolving cycle of hyperglycemia-stimulated AGE generation leading to RAGE activation may mediate ongoing production of AGE.

S100/calgranulins

S100/calgranulins are a family of >20 polypeptides characterized by prototypical EF-hand domains in their structures. Although not all S100/calgranulins are likely to bind RAGE, at least certain members of this family bind this receptor, such as but not limited to S100B, S100A12, S100A6, and S100P [14]. In addition to implications for S100/calgranulins in the pathogenesis of pro-inflammatory pathways based on a plethora of studies indicating their upregulation in immune/inflammatory tissues, S100/calgranulins, at least in part via RAGE, also contribute to tumor migration and metastases. Compelling studies indicate that S100P, via RAGE signaling, mediates tumor cell proliferation and migration. Experiments using soluble (s) RAGE, anti-RAGE antibodies and introduction of DN RAGE into tumor cells greatly suppressed these effects of S100P compared to control treatment [15].

High Mobility Group-Box 1 (HMGB1)

HMGB1 plays multiple roles within the cell and its environment. As a nuclear protein, it functions as non-histone DNA binding protein, but in cell stress or stimulation, HMGB1 may be released from cells or expressed at the leading edge of migrating cells [4]. Studies revealing that HMGB1 may be released from necrotic, but not apoptotic cells, indicated that once released into sites of injury, HMGB1 may, via autocrine and/or paracrine mechanisms, interact with RAGE [16]. In initial studies, HMGB1-RAGE interaction was shown to

mediate neurite outgrowth of embryonic rat cortical neurons [4]. In C6 glioma tumor cells, ligand-RAGE interaction stimulated cell proliferation, migration and production of active matrix metalloproteinases (MMPs), in parallel with activation of mitogen-activated protein (MAP) kinases [5]. HMGB1 and RAGE are expressed in multiple human tumors. Recently, Ellerman and colleagues summarized current knowledge regarding links of HMGB1-RAGE to human tumor stage and prognosis. For example, increased HMGB1 and/or RAGE expression have been found in human breast, colon, melanoma, prostate (especially in metastatic disease), pancreatic cancer, gastric cancer and hepatocellular carcinoma [17].

Amyloid- β Peptide (A β) and β -sheet fibrils

The discovery that RAGE bound A β (1–40 and 1–42) launched RAGE into pathways linked to neurodegeneration (Alzheimer's disease) and amyloidoses [6]. *In vitro* studies supported the binding of A β to immobilized sRAGE and studies in RAGE-bearing cells indicated that blockade of RAGE mitigated the effects of A β on cell stress and upregulation of damage-provoking pathways. Studies have been published using animal models in which RAGE/RAGE function has been altered in transgenic mice expressing mutant amyloid precursor protein (mAPP). In those animals in which RAGE was overexpressed in central nervous system neurons in the transgenic mAPP background, upregulation of neuronal RAGE augmented inflammation, reduced acetylcholinesterase positive neurites and amplified cognitive dysfunction. In contrast, blockade of RAGE signaling, via introduction of DN RAGE in neurons, mitigated cell stress and cognitive dysfunction in the mAPP background [18].

The Ligand Families of RAGE– Formation of “Compound Ligands” & First Insights into RAGE Structure

It is well-established that in diseased tissue, multiple RAGE ligand families may be represented. In murine atherosclerosis, in addition to AGE epitopes, increased expression of S100/calgranulins and HMGB1 has been noted in aorta [11]. In the db/db kidney, a model of type 2 diabetes, extensive deposition of AGEs has been noted in both glomerular and tubular structures [19]. Within both regions, S100/calgranulin-bearing inflammatory cells also infiltrate the diabetic kidney.

Beyond the single ligand, modifications within RAGE ligands may create more complex ligand structures. For example, glycation of β_2 -microglobulin, to yield AGE- β_2 -microglobulin creates a fibrillar amyloid structure (that is glycated as well) which binds RAGE and, perhaps, contributes to amyloid disease in the joints and tissues consequent to long-term renal failure [20]. Additional examples of compound ligand formation were identified from the observation that S100 proteins may be post-translationally modified by CML-AGE adducts [21]. Thus, it would not be surprising if compound ligands may form given their intimate proximity to each other in the pro-inflammatory and pro-oxidant microenvironments of chronic disease.

In recent years, first insights into the structural biology of RAGE are emerging suggesting that distinct ligands may bind to unique components of the extracellular domain of RAGE, including binding to the V-type Ig domain and/or the C-type Ig domains. For example, the binding of RAGE to the various members of the S100/calgranulin family has led to novel insights into RAGE structure and the precise domains and mechanisms through which specific S100-sRAGE interactions occur. Ostendorp and colleagues studied S100B in detail and found through analysis of the X-ray structure of human S100B that this molecule existed in an octameric architecture with four homodimeric units arranged as two tetramers in a tight array. Binding studies revealed that the tetrameric S100B bound RAGE with higher affinity than dimeric S100B and by analytical ultracentrifugation, the binding of

S100B tetramer to RAGE was shown to occur through binding to RAGE V-domain [22]. Xie and colleagues studied S100A12 through high resolution NMR spectroscopy and showed that S100A12 bound to the first C-type immunoglobulin domain of RAGE. Native gel electrophoresis established that sRAGE forms tetramers that bind to hexamers of calcium-S100A12 complexes [23]. Further evidence for the diversity of S100/calgranulin binding to distinct domains of RAGE was shown by Leclerc and colleagues. These authors showed that S100B bound to the V-type and C1-immunoglobulin domains of RAGE, but S100A6 specifically interacted with the C1- and C2-immunoglobulin domains of RAGE [24].

Recently, Xie and colleagues examined mechanisms of interaction of AGEs with RAGE. Preparations of AGE-BSA bound to the V-domain of RAGE, but single amino acid modifications such as CML or CEL (carboxy ethyl lysine) failed to bind to RAGE. By in-cell fluorescence resonance energy transfer (FRET), the investigators showed that at the cell surface, RAGE is a constitutive oligomer and suggested that such a structure is important for RAGE to recognize patterns of AGE-modified proteins with affinities less than 100 nM [25].

Such structural insights will form the basis of understanding how compound ligands may engage RAGE and if they do so in a manner common to either of the ligand components alone. These considerations will be important in optimal drug design aimed at blocking RAGE ligand-RAGE interaction, as we predict in chronically inflamed and aged tissue, multiple RAGE ligands are likely to accumulate and play roles in pathology.

In the sections to follow, we focus on the biology of RAGE in chronic disease settings. From data in experimental model systems, it is clear that RAGE-dependent signal transduction is an essential factor in mediating the biological impact of RAGE's diverse ligand families.

RAGE & Cardiovascular Stress

Insights from Studies in Human Subjects

RAGE and its ligand expression have been evaluated in human cardiovascular disease, particularly in diabetes. Diabetic subjects are more vulnerable to accelerated atherosclerosis and cardiac events compared to age-matched subjects without diabetes. In atherosclerotic lesions from diabetic subjects who died suddenly from cardiovascular complications, increased expression of RAGE and one of the S100/calgranulins (S100A12) was demonstrated. RAGE expression was noted in apoptotic SMC and macrophages within the lesions [26]. Based on these and other findings, biomarkers and mediators of RAGE action in the cardiovascular system have been sought as a means to gauge the extent of atherosclerosis or the risk of complications from the disease. In this context, at least two general areas of RAGE biomarkers are under study by multiple groups.

First, soluble levels of RAGE are detectable in the plasma of human subjects. Distinct mechanisms may underlie this formation of sRAGE; specifically evidence suggests that splice variants of RAGE producing "endogenous secretory or esRAGE" (also known as RAGEv1) as well as proteolytic cleavage of cell surface RAGE may account for the detection of plasma sRAGE [27–29]. In the case of esRAGE/RAGEv1, specific antibodies may distinguish esRAGE/RAGEv1 from total sRAGE due to a unique stretch of amino acids identified in this construct. Hence, generation of antibodies targeting only those unique amino acids has proved useful in identification of esRAGE/RAGEv1 vs. total sRAGE. Studies in human subjects are now emerging suggesting potential relationships between "sRAGE" and cardiovascular diseases; these studies, however, are far from conclusive. For

example, Falcone and colleagues first studied total sRAGE in nondiabetic men and reported that levels of plasma sRAGE were significantly lower in subjects with angiographically-proved coronary artery disease than those without the disease [30]. Other studies suggested that levels of esRAGE were inversely correlated with carotid artery intima/media thickness in subjects with type 2 diabetes [31]. In end-stage renal disease, low circulating levels of esRAGE predicted cardiovascular mortality [32]. In that study, however, the authors noted that esRAGE levels and cardiovascular disease were confounded by the variables of age and diabetes. Clues to potential mechanistic links between tracking sRAGE and cardiovascular pathology were suggested by the work of Nakamura and colleagues who showed that serum levels of sRAGE were positively associated with markers of inflammation (MCP-1 and TNF- α) in subjects with type 2 diabetes [33].

In addition to probing levels of circulating sRAGE, *RAGE* gene polymorphisms are being studied in human subjects for their potential relationship to cardiovascular disease and diabetes. Among these polymorphisms of *RAGE* is the -374T/A polymorphism of the *RAGE* gene promoter. Falcone and colleagues showed that in a group of Caucasian patients with angiographically-proved coronary artery disease, the mean number of diseased vessels was significantly lower in patients with the AA vs. the AT or TT genotype [34]. These same authors studied the potential utility of this polymorphism as a prognostic marker in subjects with coronary artery disease. They found that in a series of 643 subjects who underwent myocardial revascularization, over a period of 4.2 years, the AA genotype was significantly related to a better outcome in non-diabetic subjects vs. AT or TT [35]. However, no such association was evident in the diabetic subjects in this cohort.

Other variants of the *RAGE* gene have been assessed in cardiac disease. Hofmann and colleagues found no association of the *RAGE* G82S polymorphism with the incidence of cardiovascular disease in the Framingham offspring study [36].

Taken together, studies are emerging in human subjects examining potential links to *RAGE* and sRAGE in cardiovascular disease, but larger studies across multiple populations and groups are clearly required. Indeed, such efforts are rational, as studies in animal models support roles for *RAGE* in atherosclerosis in the presence and absence of diabetes.

RAGE and Animal Models of Atherosclerosis & Restenosis – Making the Case for Antagonism of RAGE

A number of studies have explored the role of *RAGE* in animal models of cardiovascular disease. In rodents, neointimal expansion consequent to distinct injuries, such as hyperglycemia, hyperlipidemia or guide-wire injury to the blood vessel wall, has been addressed using both pharmacological and gene-modification strategies to target *RAGE*.

Murine models were developed to test the concept that diabetes accelerates vascular inflammation and atherosclerotic lesion area development and progression in apolipoprotein E null mice. These mice, a model of spontaneous hypercholesterolemia which develops on normal chow diet, displayed significantly increased atherosclerotic lesion area and inflammation when rendered either type 1 or type 2-like diabetic [37–38]. In the former case, the β -cell toxin streptozotocin (stz) was used to induce hyperglycemia, and in the latter case, apolipoprotein E null mice were bred into the insulin-resistant db/db background (deficient leptin receptor signaling leading to obesity, insulin resistance and hyperglycemia). In both models of diabetes, expression of *RAGE* and its inflammatory ligands was increased in the aortas of diabetic vs. non-diabetic apolipoprotein E null mice. An important caveat in interpretation of these models, however, is that in parallel with increased levels of glucose and atherosclerosis, diabetic mice displayed increased plasma lipid levels, thereby blurring to some extent the specific etiologic factors underlying accelerated atherosclerosis that were

independent of this classical and established risk factor. However, in recent studies, transgenic mice expressing the first and chief enzyme of the polyol pathway, aldose reductase, displayed increased atherosclerosis *independent* of lipid status when rendered diabetic with stz [39]. Such new models provide more precise tools to dissect the specific effect of *glucose and its consequences* in vascular pathology.

Notwithstanding these caveats, however, the diabetic apolipoprotein E null mouse provided a model to test the biological impact of RAGE in vascular pathology. In the first studies, the extracellular ligand-binding domain of RAGE (sRAGE) was expressed in a baculovirus expression system, purified to homogeneity, and devoid of possible contamination with lipopolysaccharide. Based on pharmacokinetic studies, sRAGE was administered once daily to diabetic mice by intraperitoneal route beginning immediately at the first evidence of hyperglycemia. Compared to vehicle treatment in diabetic mice, those mice receiving sRAGE revealed a significant decrease in atherosclerosis without changes in glucose, lipid or lipid profile. Analysis of the aortas of the various animal groups revealed that multiple markers of inflammation, although increased in the diabetic state, were reduced by sRAGE treatment [37].

When sRAGE was instituted later in the course of diabetes in apolipoprotein E null mice, compared to vehicle treatment, those mice receiving sRAGE displayed stabilization of lesion area and reduction in vascular inflammation [40]. Most notably, the dramatic increase in complexity of the atherosclerotic lesions was prevented in diabetic mice treated with sRAGE from age 14 to 20 weeks. Taken together, these experiments were the first to suggest roles for RAGE in diabetic atherosclerosis. Of note, upregulation of RAGE and its ligands was identified in non-diabetic mice vasculature as well, albeit to lesser degrees than that found in diabetic animals. Consistent with pathogenic roles for ligand-RAGE interaction as a contributing mechanism to atherosclerosis in the absence of diabetes, administration of sRAGE reduced atherosclerotic lesion area and inflammation in non-diabetic apolipoprotein E null mice [38,40].

To complement studies using sRAGE, two recent studies have assessed atherosclerosis in apolipoprotein E null mice bred into the RAGE null background. In both the presence and absence of diabetes, deletion of RAGE mitigated vascular inflammation and reduced atherosclerotic lesion area [11,41]. As RAGE was upregulated in the endothelium at an age *preceding* the development of frank atherosclerotic plaques in apolipoprotein E null mouse aorta, non-diabetic apolipoprotein E null mice were bred into a background of transgenic mice in which the cytoplasmic domain of RAGE was deleted in EC (as driven by the pre-endothelin 1 promoter) to introduce DN RAGE [11]. Atherosclerosis was significantly reduced in those mice, and levels of adhesion molecules, chemokines and inflammation-modulating cytokines were reduced by deletion of RAGE signaling in endothelium in the apolipoprotein E null background.

Endothelial dysfunction as a prime mechanism underlying atherosclerosis in this model, at least in part due to RAGE, was underscored by experiments in which aortic rings were retrieved from the aortas of apolipoprotein E null mice, apolipoprotein E null/RAGE null mice and apolipoprotein E null/transgenic PPET DN RAGE mice. In the absence of diabetes, aortic rings retrieved from mice devoid of RAGE or endothelial RAGE signaling in the apolipoprotein E null background displayed enhanced vasorelaxation responses to acetylcholine compared to apolipoprotein E null mice [11]. To further dissect the role of RAGE and RAGE signaling in the endothelium, aortic ECs were retrieved from C57BL/6, RAGE null and transgenic PPET DN RAGE mice. Exposure of wild-type C57BL/6 ECs to RAGE ligand S100B or AGE-containing oxidized LDL resulted in upregulation of Vascular Cell Adhesion Molecule-1 and MMPs. In contrast, when ECs from RAGE null or transgenic

PPET DN RAGE were treated with these ligands, VCAM-1 and MMP responses were markedly reduced. Key roles for JNK MAP kinase signaling in transducing the effects of RAGE ligands on upregulation of VCAM-1 in ECs were elucidated by experiments in which wild-type ECs were subjected to pharmacological inhibitors of JNK or RNA interference (RNAi) to reduce JNK expression. In those studies, compared to respective controls, treatment with S100B or oxLDL failed to upregulate VCAM-1, whereas inhibitors of P44/p42 MAP kinase had no effect [11].

As RAGE is expressed in distinct vascular cells (SMCs) and inflammatory cells (macrophages and T lymphocytes), more in-depth study of the role of RAGE in each cellular compartment is essential to uncover potentially beneficial roles for RAGE signaling in vascular pathology. Although the bulk of the evidence would suggest that in vascular diseases, blocking RAGE prevents or delays cardiovascular injury, recent evidence suggesting important roles for RAGE in T lymphocyte priming and DC maturation and homing suggest adaptive roles for RAGE in immune responses to environmental challenges. If and how such considerations may be relevant to cardiovascular disease must continue to be investigated.

In this context, a distinct model of vascular injury, that induced by endothelial denudation in the vessel wall, revealed roles for RAGE and its ligands in aberrant repair responses, particularly in SMCs. Clues to potential roles for RAGE in smooth muscle responses *in vivo* were deduced from earlier studies in *in vitro* models. In rabbit SMCs, incubation with AGE-BSA mediated smooth muscle chemotaxis [42]. Although the precise receptors mediating the effects of AGE-BSA were not studied in that work, it was shown that antibodies to TGF- β suppressed the stimulatory effects of AGE-BSA. When aortic SMCs cultured from non-diabetic rats were exposed to sera from type 1 diabetic (stz) rats, increased smooth muscle growth (proliferation) resulted, in a manner suppressed by antibodies to AGE or to RAGE. Further, *in vitro*-prepared AGE-BSA stimulated vascular smooth muscle growth in a RAGE-dependent manner [43].

Multiple signal transduction pathways have been linked to RAGE in SMCs. AGE-RAGE interaction activates p21ras and p44/42 MAP kinase in a manner dependent on oxidative stress, and key roles for Src kinase in mediating the impact of S100B in vascular SMCs were shown [44–45]. In the latter study, nucleofection of a Src DN mutant or caveolin-1 mutant lacking the scaffolding domain or cav-1 short hairpin RNA reduced S100B-mediated inflammatory gene expression. Shaw and colleagues tested if S100B augmented smooth muscle responses to a distinct stimulus highly relevant to vascular pathology, angiotensin II. These authors showed that S100B, through RAGE, augmented angiotensin II-mediated tyrosine phosphorylation of Jak2 and, in parallel, smooth muscle proliferation [46].

These concepts have been tested in both diabetic and non-diabetic animals. Diabetic Zucker fatty rats subjected to balloon injury to the carotid artery displayed increased SMC proliferation and neointimal expansion (assessed by Intima/Media [I/M] ratio) compared to non-diabetic age- and gender-matched control animals [47]. When diabetic rats were treated with sRAGE, beginning immediately after balloon injury, a significant decrease in I/M ratio was reported compared to vehicle treatment. Measures of smooth muscle proliferation in the expanding neointima suggested that RAGE blockade reduced this proliferative response.

In order to establish evidence for the signaling pathways mediating the effects of RAGE on smooth muscle proliferation and/or migration in response to *in vivo* injury, experiments in murine models were performed [48]. Non-diabetic mice were subjected to femoral artery endothelial denudation as induced by guide wire. Compared to very low levels of RAGE and two of its pro-inflammatory/pro-oxidative ligand families, AGEs and S100/calgranulin in

the uninjured vessel, arterial injury resulted in a significant increase in RAGE, AGE and S100/calgranulin in the vessel wall and expanding neointima. Administration of sRAGE significantly reduced I/M ratio compared to vehicle treatment. Similarly, experiments in RAGE null mice revealed marked protection from neointimal expansion vs. littermate controls.

As the principal site of expression of RAGE in the injured vessel wall was in SMCs, a novel transgenic mouse was developed to drive expression of DN RAGE specifically in SMCs, under control of the SM22 α promoter. In these animals, compared to their wild-type littermates, significantly reduced neointimal expansion was noted in response to endothelial denudation. Messenger RNA transcripts encoding tenascin C and MMP12 were markedly reduced in sRAGE neointima vs. vehicle neointima, and trichrome staining revealed reduction in collagen and matrix production at the site of injury. [48]

To identify the signal transduction pathways linked to arterial injury via RAGE, lysates were prepared immediately after endothelial denudation in wild-type mice. Increased phosphorylation of Jak2, Stat3, p44/p42 MAP kinase and protein kinase B was noted; in the sRAGE-treated animals, only phosphorylation of Jak2/Stat3 was reduced, thereby implicating key roles for this pathway in RAGE-mediated signaling in the injured vessel. These concepts were addressed in SMCs retrieved and cultured from the aortas of wild-type, RAGE null and transgenic SM22 α DN RAGE mice. Upon stimulation with S100B, Jak2/Stat3 phosphorylation, migration and proliferation were significantly increased in wild-type, but not the RAGE or RAGE signaling deficient SMCs [48]. An inhibitor of Jak2 (AG490) had similar suppressive effects on RAGE ligand (S100B)-mediated SMC properties.

Distinct studies suggested potential links to RAGE and peroxisome proliferator-activated receptor γ (PPAR γ) in responses to rat carotid artery injury. *In vitro* (SMCs treated with S100/calgranulins) and *in vivo* (rat balloon injury), treatment with rosiglitazone resulted in decreased expression of RAGE and smooth muscle proliferation. *in vivo*, this treatment reduced neointimal expansion. Consistent with the premise that the actions of rosiglitazone were in part through regulation of RAGE, administration of rosiglitazone and sRAGE resulted in no further attenuation of I/M ratio [49]. Such studies suggest hierarchical relationships between RAGE signaling and PPAR γ nuclear signaling.

Taken together, these findings indicate that RAGE signaling in the vessel wall importantly modulates responses to two diverse forms of injury, hyperlipidemia and physical injury. In other studies, RAGE has been shown to mediate regulation of tissue-damaging genes in hypoxia, such as Early growth response-1 (Egr-1). Via PKC β II-dependent mechanisms, RAGE action in ECs signals upregulation of Egr-1, at least in part through JNK MAP kinase signaling [50]. These findings likely underlie the deleterious effects of RAGE in ischemia/reperfusion injury in organs such as the heart and liver.

In addition to large vessel disease, the biology of RAGE is implicated in the classical “microvascular” complications of diabetes. Here, we will consider the findings linking RAGE to human and murine diabetic nephropathy and neuropathy.

RAGE & the Microvascular Complications of Diabetes

Diabetic Nephropathy

Multiple studies indicate that RAGE is expressed at low levels in the human kidney in homeostasis, and that its expression is increased in diseases of the kidney, including diabetes [51–54]. In human diabetic kidney, RAGE is particularly expressed in podocytes and in vascular cells [51]. The ligands of RAGE accumulate in diabetic kidney; in particular

increased AGE products have been shown compared to non-diabetic controls throughout the glomerular and tubular structures [51]. In advanced kidney disease, such as in subjects receiving chronic hemodialysis therapy, increased levels of sRAGE were observed [55]. It is not clear if such high levels of sRAGE were related to decreased clearance and/or stimulation by environmental toxic factors. An additional explanation is that sRAGE levels may be influenced by genetic factors. For example, Kalousova and colleagues reported that in subjects with end-stage renal disease receiving hemodialysis, the highest sRAGE levels were found in individuals with the -429 CC and 2184 GG polymorphisms of the *RAGE* gene. [56]

In this context, multiple polymorphisms of the *RAGE* gene have been studied in relation to diabetic kidney disease [57–63]. Kankova and colleagues performed a haplotype analysis of the *RAGE* gene and reported that the haplotype containing minor alleles in positions -429 and 2184 were associated with diabetic nephropathy in type 2 diabetes, and with earlier onset of nephropathy [63]. This particular study addressed multiple known SNPs in the *RAGE* gene (-429T/C, -374T/A, G82S, 1704G/T, 2184A/G and 2245G/A) in 605 Caucasian subjects.

Of note, there is evidence that levels of sRAGE may be modulated by therapeutic intervention in subjects with renal complications of diabetes. Type 1 diabetic subjects treated with the angiotensin-converting enzyme inhibitor perindopril displayed increased levels of sRAGE after treatment; the authors speculated that perhaps such therapy reduced AGE accumulation in plasma, in part by increasing the production and secretion of sRAGE [64].

In addition to supportive evidence linking RAGE to human diabetic nephropathy, multiple experiments in animal models have supported roles for RAGE in diabetic kidney disease. In the first study, obese diabetic db/db mice were treated with long-term sRAGE therapy. At the later stages of disease at age 27 weeks, sRAGE-treated db/db mice displayed less albuminuria and mesangial expansion compared to vehicle-treated diabetic mice [19]. In C57BL/6 mice bred into the RAGE null background and rendered diabetic with stz, early indices of mesangial expansion and albuminuria were reduced compared to RAGE-expressing diabetic animals. By Northern blotting, cortex levels of VEGF and TGF- β transcripts were reduced in RAGE null vs. wild-type mice [19]. A later study supported the role of RAGE in diabetes nephropathy using a distinct model of RAGE deficiency. In that study, significant suppression of the development of kidney enlargement, increased glomerular number, mesangial expansion, advanced glomerulosclerosis, increased albuminuria and creatinine clearance were reduced by RAGE deletion (in a gene dosage-dependent manner) [65].

Others have developed anti-RAGE neutralizing antibodies for testing in animal models of types 1 and 2 diabetes. In both cases (db/db and stz), administration of anti-RAGE antibody reduced the renal structural and pathological changes observed in the control diabetic animals [66–67]. Interestingly, in stz diabetic mice, the upregulation of connective tissue growth factor (CTGF) and collagen IV alpha 1, which were upregulated in the control diabetic animals, were not reduced in those animals receiving antibodies to RAGE. We deduce the following possible explanations for such findings: these pathways are not relevant directly to diabetic nephropathy (in the mouse), the time course was not reflective of the pathology, or the protein/activity levels of these factors did not match the mRNA transcript levels.

Taken together, extensive data have been generated both in the human and in the rodent to support that RAGE plays important roles in the pathogenesis of diabetic nephropathy. What

is not clear from these studies, however, is what the precise cell type(s) are that contribute to the development of renal injury in diabetes. To address the general role of RAGE in albuminuria and glomerulosclerosis, Guo and colleagues tested the role of RAGE in doxorubicin (adriamycin)-induced nephropathy. In this model, a swift and progressive form of glomerular disease with severe podocyte effacement, albuminuria and glomerulosclerosis develop. When doxorubicin-treated mice were studied, rapid upregulation of mRNA transcripts for RAGE were noted in the kidney. The key cell type expressing RAGE post-doxorubicin in the kidney was the podocyte, and not other glomerular or tubular cells. Administration of sRAGE to doxorubicin-treated wild-type BALB/c mice, or treatment of RAGE null (BALB/c) mice with doxorubicin resulted in dramatic but not 100% percent protection from glomerular injury.

In cultured murine podocytes, administration of doxorubicin or RAGE ligand S100B caused rapid oxidative stress and upregulation of mRNA transcripts encoding TGF- β and CTGF in a manner suppressed by sRAGE, anti-RAGE IgG or introduction of DN RAGE [68]. Of note, other studies have implicated RAGE in podocyte expression of MCP-1 [69].

Future studies must further explore the role of RAGE in the podocyte in the pathogenesis of diabetic nephropathy – from mouse to the human model. As others have reported RAGE expression and function in tubule cells [70], it is clear that the full spectrum of RAGE in the kidney needs further analysis.

Diabetic Neuropathy

Studies in human subjects with diabetic peripheral neuropathy have confirmed that RAGE is expressed in sural nerve, together with RAGE ligands, particularly in the microvasculature. In parallel, increased expression of activated NF- κ B p65 and IL-6 co-localized with RAGE in this tissue [71]. Wada and colleagues observed deposition of AGE in diabetic peripheral nerve and in serum of diabetic patients. Those investigators found that RAGE was expressed in endothelial and Schwann cells in the diabetic peripheral nerve [72]. The effects of islet transplantation on peripheral nerve markers of injury were studied in subjects with type 1 diabetes who underwent islet transplantation concurrent with kidney transplantation. Compared to subjects receiving solely kidney transplants, those receiving islet/kidney transplantation displayed improved nerve conduction velocity score, and lower levels of AGE and RAGE in the perineurium and vaso nervorum of skin biopsies [73]. To date, at least one study has examined levels of sRAGE and esRAGE in type 2 diabetic subjects in neuropathy. When patients were stratified for no, mild, moderate or severe deficits in neuropathy or disability score, there were no correlations with sRAGE or esRAGE noted [74].

Studies in experimental animal models have used distinct strategies to test the role of ligand-RAGE in the pathogenesis of neuropathy. Compared to wild-type mice with diabetes, RAGE null mice with comparable degrees of hyperglycemia displayed decreased loss of pain perception, without changes in PGP0.5 positive plantar fibers. In parallel, administration of sRAGE to wild-type diabetic mice prevented loss of pain perception. Prominent suppression of activated NF- κ B was observed in the presence of RAGE blockade/deletion [71].

Toth and colleagues found similar protective roles for RAGE deletion in diabetic neuropathic features in a mouse model (stz diabetic Swiss Webster mice). RAGE expression increased in a time-dependent progressive manner in diabetic vs. control mice in peripheral epidermal axons, sural axons, Schwann cells, and sensory neurons within ganglia. Electrophysiological and structural abnormalities were observed in wild-type diabetic mice but were attenuated in RAGE null mice with long-term diabetes (up to 9 months). In

parallel, activation of injury-linked signaling pathways (NK- κ B and Protein Kinase C β II) were reduced in RAGE null mice [75].

RAGE biology in the peripheral nervous system is complex, however, and much more needs to be learned to elucidate the precise function of RAGE in injury vs. repair processes [76–77]. In non-diabetic mice subjected to unilateral crush injury to the sciatic nerve, administration of sRAGE or blocking antibodies to RAGE, HMGB1 or S100/calgranulin resulted in suppression of repair, in that motor and sensory nerve conduction velocities were reduced, as were myelinated fiber densities [76]. Both inflammatory and axonal contributions of RAGE signaling were required for effective repair, as transgenic mice expressing DN RAGE in either axonal elements (thy-1 promoter) or cells of mononuclear phagocyte lineage (scavenger receptor-type A promoter) displayed diminished regeneration compared to wild-type littermates [77]. These findings strongly suggest innate roles for RAGE in inflammatory and axonal responses to acute nerve injury in the absence of diabetes. What is the situation in the diabetic nerve, however, where chronic upregulation of RAGE and its ligands is present at baseline? Studies are ongoing to elucidate the precise pathways by which acute injury to the non-diabetic and diabetic peripheral nervous system is impacted by RAGE signaling.

The Ligand-RAGE Axis: Enveloping Diabetes from Initiation to Complications

Clues to seminal roles for RAGE in the inflammatory response were deduced from the tissue distribution of RAGE. In addition to neutrophils [78], RAGE is expressed in monocytes/macrophages [79], T and B lymphocytes [80] and DCs [81]. RAGE mediates ligand-stimulated chemotaxis and haptotaxis of macrophages. In addition to direct stimulation of RAGE on these cells, is evident that when activated (migration and production of cytokines and MMPs), inflammatory cells may subsequently release HMGB1 and S100/calgranulins, thereby propagating inflammatory responses at least in part via RAGE [16,82–84]. Thus, monocytes/macrophages may be lead actors in the initiation and propagation of RAGE-dependent roles in inflammation.

Beyond monocytes/macrophages, RAGE plays central roles in lymphocyte migration and priming. Murine models of diseases linked to adaptive immunity illustrated the link to RAGE. In addition to delayed type hypersensitivity [3], colitis [3], arthritis [85] and orthotopic transplantation [86] as examples, induction of experimental autoimmune encephalomyelitis (EAE) in mouse models of multiple sclerosis was found to depend importantly on RAGE. RAGE blockade suppressed EAE induced by myelin basic protein (MBP) peptide or encephalitogenic T lymphocytes, or when EAE occurred spontaneously in T cell receptor (TCR)-transgenic mice devoid of endogenous TCR- α or TCR- β chains. RAGE signaling in T lymphocytes (CD4) was essential in lymphocyte migration into the murine CNS, as mice expressing DN RAGE in T lymphocytes (CD4) were resistant to MBP-induced EAE [87].

Two recent studies support important roles for RAGE biology in the pathogenesis of type 1 diabetes in murine models. Studies in NOD/*scid* mice subjected to adoptive transfer of diabetogenic splenocytes from NOD mice revealed that compared to vehicle administration, treatment with sRAGE increased the time to development of T1D. In parallel, expression of pro-inflammatory cytokines in the islets, including TNF- α and IL-1 β , were reduced in the islet [80]. In studies using RAGE null mice, compared to wild-type animals, RAGE null mice rendered diabetic with stz displayed delayed rejection of islet allografts [88].

The effects of RAGE on T lymphocyte proliferation were studied in murine and human donor-reactive T lymphocyte priming studies. Incubation of murine peripheral blood mononuclear cells with sRAGE or treatment with blocking antibodies to RAGE resulted in a

significant decrease in lymphocyte proliferation versus control-treated cultures [86,88]. Similar results were found in human lymphocytes stimulated by alloresponses [86].

Essential roles for RAGE in T lymphocyte priming were revealed in studies in which transfer of RAGE-deficient OT II cells into OVA-immunized hosts resulted in reduced proliferative responses, that were even further reduced when these cells were infused into RAGE null hosts [89]. The latter studies suggest that RAGE-expressing host cells such as endothelial cells, B lymphocytes and/or DCs contribute to the host response in RAGE-expressing recipients. Studies are in progress to rigorously address the relevant compartments in the recipients.

Summary

Collectively, these findings suggest the emerging concept that RAGE's roles in the immune response are diverse and situation-specific – from innate responses in cecal ligation and puncture-induced sepsis [90] - to the adaptive immune response in T lymphocyte priming and allotransplantation responses [86,88–89]. These concepts lead to the intriguing possibility that in diabetes (particularly in type 1 diabetes) the factors that signal autoimmunity and β -cell destruction, at least in part via RAGE, are the same mediators that subsequently attack the target organs in complications from the micro- to the macrovessel; processes which then feed-back to *further* damage RAGE-expressing β -cells (figure 1).

Disentangling the complex cellular relationships from the earliest stages of autoimmune attack of the pancreatic β -cell to the failure of repair in ravaged diabetic tissues will likely reveal seminal roles for RAGE in the pathogenesis of diabetic complications. Studies to date suggest that blocking RAGE largely restores beneficial adaptations to macro- and microvessel assault in this disease. Further, experiments in cecal ligation and puncture, massive liver resection and infection with *Listeria monocytogenes* [81,90,91] in mice suggest that RAGE exaggerates innate responses throughout recruitment of the adaptive immune system and that its blockade draws the organism back toward homeostasis. The situation in the human may be more complex, and thus the challenge in identifying optimal therapeutic strategies will be to harness the adaptive impact of RAGE in immunity and inflammation - without rendering the host vulnerable to acute biological or environmental challenges.

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1. . The ligand-RAGE axis- initiation and potentiation of pancreatic β -cell damage: from autoimmunity to diabetic complications

RAGE was first discovered as a signal transduction receptor for Advanced Glycation Endproducts (AGEs). The identification of RAGE's multi-ligand nature, and its intimate role in the inflammatory response, however, places RAGE at multiple points in the pathogenesis of type 1 diabetes – and its end-organ damage. (Auto)immune attack at the pancreatic β -cell leads to infiltration of inflammatory cells and increased expression/release of S100/calgranulins and HMGB1. Such ligands target RAGE in the islet and contribute to destruction and, ultimately, hyperglycemia. Hyperglycemia triggers rapid generation of AGEs, thereby recruiting RAGE in target organs via the micro- and macrovasculature. One component of RAGE's action in target organs is the infiltration of inflammatory cells as well as end-organ stress – processes leading to release of S100/calgranulin and HMGB1. These ligands, together with AGEs, damage such cells as podocytes, endothelial and smooth muscle cells, Muller cells of the retina, cardiomyocytes, and central and peripheral nervous system neurons. Chronic inflammatory signaling, may, we predict, feed back to further damage the pancreatic islet and eliminate the possibility of regeneration. Stopping the cycle of RAGE action in inflammation and diabetes may be a novel form of therapy in this disorder and its complications.