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The Multiple Faces of RAGE - Opportunities for Therapeutic Intervention in Aging and Chronic Disease

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Abstract

Introduction—This review focuses on the multi-ligand receptor of the immunoglobulin superfamily, receptor for advanced glycation endproducts (RAGE). The accumulation of the multiple ligands of RAGE in cellular stress milieu links RAGE to the pathobiology of chronic disease and natural aging.

Areas Covered—In this review, we present a discussion on the ligands of RAGE and the implications of these ligand families in disease. We review the recent literature on the role of ligand-RAGE interaction in the consequences of natural aging; the macro- and microvascular complications of diabetes; obesity and insulin resistance; autoimmune disorders and chronic inflammation; tumors and Alzheimer's disease. We discuss the mechanisms of RAGE signaling through its intracellular binding effector molecule, the formin DIAPH1. Physico-chemical evidence by which the RAGE cytoplasmic domain binds to the FH1 (formin homology 1) domain of DIAPH1, and the consequences, is also reviewed.

Expert Opinion—We discuss the modalities of RAGE antagonism currently in pre-clinical and clinical studies. Finally, we present the rationale behind potentially targeting the RAGE cytoplasmic domain-DIAPH1 interaction as a logical strategy for therapeutic intervention in the pathological settings of chronic diseases and aging in which RAGE ligands accumulate and signal.

Keywords

receptor; diabetes; obesity; inflammation; Alzheimer's disease; neurodegeneration

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1.0. Introduction: RAGE & its Diverse Ligand Families

The multi-ligand immunoglobulin superfamily molecule, receptor for advanced glycation endproducts (RAGE), was discovered on account of its ability to bind the advanced glycation endproducts (AGEs), the products of nonenzymatic glycation and oxidation of proteins and lipids that occurs at slow rates in normal health and aging, but to accelerated degrees in diabetes [1–4]. AGEs are a heterogeneous group of post-translationally modified species, including adducts such as carboxy methyl lysine (CML), carboxy ethyl lysine (CEL), pentosidine, pyralline and MOLD/GOLD, to indicate a few. Although RAGE was identified as a binding molecule for the heterogeneous AGEs, one of these AGEs, CML-AGE, is a specific AGE ligand of RAGE [5]. Beyond endogenous formation of AGEs through pre-AGEs such as glyoxal, methylglyoxal (MG) and 3-deoxyglucosone (3-DG), AGEs such as CML-AGE may also gain access to the body through the diet [6]. RAGE may contribute to the propagation of MG-derived AGE products. In diabetic kidney and diabetic retina, lower levels of the pre-AGE MG were noted in diabetic mice devoid of *Ager* (gene encoding RAGE) versus the wild-type diabetic controls, despite equal degrees of hyperglycemia. Further analysis uncovered that RAGE-dependent downregulation of glyoxalase1 (GLO1) contributed to these findings [7, 8].

Beyond the AGEs, RAGE is also a signaling receptor for certain members of the S100/calgranulin family and high mobility group box 1 (HMGB1), amyloid-beta (β) peptide and β -sheet fibrils, lysophosphatidic acid (LPA), Mac-1 and phosphatidylserine [9–14]. These diverse classes of ligands suggest strong links to the metabolic state; both glucose- and lipid-modified species may bind RAGE, thereby implicating their interaction with RAGE as a contributing mechanism in metabolic disorders. Table 1 provides a review of the most-commonly studied RAGE ligands.

In human subjects, a rich literature has emerged on the testing of the associations of levels of soluble RAGEs in plasma or serum with the presence or absence of chronic disease states, as well as the severity of disease [15–17]. Essentially, there are two known forms of soluble RAGEs. Cell surface cleavage of full-length membrane-bound RAGE by matrix metalloproteinases (MMPs) and A-Distintegrin and Metalloprotease (ADAM)-10 results in the formation of soluble RAGE or sRAGE, which is essentially the extracellular domain [18, 19]. A distinct form of soluble RAGE may be derived from pre-mRNA alternative splicing. Endogenous secretory (es) RAGE or RAGEv1 is not identical to the aforementioned sRAGE; esRAGE contains a unique span of 16 amino acids in the C-terminal region [20]. Of measurable soluble RAGE in circulation, esRAGE represents approximately 20% of the total material.

In the sections to follow, we review the main pathological settings in which RAGE has been implicated. These conditions define the likely disease states for ultimate clinical trial testing using the multiple forms of antagonists of RAGE/RAGE signaling in pre-clinical and clinical development.

2.0. Aging – the build-up of AGEs and tissue stress

Francheschi and Campus recently reviewed the phenomenon of “inflammation” [21]. Inflammaging refers to the “sterile” non-infection associated appearance of mild indices of low-grade inflammation, such as increased expression of inflammatory biomarkers including C-reactive protein (CRP) and Interleukin-6 that accompanies the aging process. In multiple studies in the elderly, significantly higher levels of these molecules serve as biomarkers for changes in energy balance and metabolic function in aging. Despite the apparent importance of this mechanism in potentially exacerbating the impact and failure of adequate resolution of aging-associated diseases, the underlying mechanisms are not fully known. One potential mechanism is the accumulation and reduced clearance of AGEs, thereby enhancing their interaction with RAGE and its downstream consequences.

In aging tissues, increased expression and activity of the first enzyme of the polyol pathway, aldose reductase (AR) has been demonstrated [22]. Increased action of the AR pathway in aging is a key mechanism for the generation of AGEs. In aged versus young Fischer 344 rats, increased expression of AR localized to endothelium and smooth muscle cells in the aorta. Impaired endothelial-dependent relaxation (EDR) in response to acetylcholine in the aged rat aorta was accompanied by increased levels of MG, which were attenuated by inhibition of AR in the rats. Further, aging-dependent impaired EDR was prevented by treatment of the rats with recombinant sRAGE, the extracellular ligand binding domains of the receptor (V-C1-C2), which binds and sequesters RAGE ligands, thereby preventing their activation of the cell surface receptor [22].

The question of whether life style intervention in experimental models and human subjects in aging might impact the AGE-RAGE axis has been studied. First, moderate intensity exercise treatment or administration of the RAGE antagonist FPS-ZM1 in aged rats reduced levels of MG and CML-AGE, in parallel with improved EDR in the aorta, reduced pulse wave velocity (PWV) and reduced multiple markers of oxidative stress [23]. Second, the effect of nine months ingestion of control or high CML-enriched diets was tested in wild-type mice and mice devoid of *Ager* [24]. Diets high in CML impaired EDR and increased PWV in the wild-type but not the *Ager* null mice, thereby reinforcing that increased levels of AGEs may drive abnormalities in the vasculature akin to that of aging, at least in part via RAGE.

Kotani and colleagues tested the effect of exercise and increased physical activity in community-dwelling elderly human subjects on levels of soluble RAGEs, using measurement of the levels of soluble RAGEs as “surrogates” of RAGE activity *in vivo*. The authors reported a reduction in levels of sRAGE after six months of an interventional program designed to increase physical activity [25]. Levels of RAGE ligands were not measured in that study; it may be speculated that perhaps lower levels of sRAGE reflected lower degrees of MMP or ADAM10 activity, although this was not directly tested.

Taken together, studies in experimental models and human subjects suggest involvement of the RAGE pathway in mechanisms of vascular aging. In the section to follow, we consider the effects of this pathway on diabetic complications.

3.0. Diabetes – AGEs, oxidative and inflammatory stress and roles for RAGE

RAGE has been linked to the pathogenesis of both the macrovascular and microvascular complications of diabetes. Increased RAGE expression in the diabetic blood vessels, target cells such as cardiomyocytes, podocytes and neurons, as well as immune cells (such as monocytes/macrophages and lymphocytes) places the receptor alongside higher levels of its ligands [2]. We surmise that the co-localization of RAGE with its ligands sets the stage for increased cellular activation and stress in the diabetic milieu. Unlike the situation in acute inflammation or infection, where the offending mediating species such as lipopolysaccharide or other toll-receptor ligands are short-lived and rapidly removed with resolution of inflammation, in diabetes, ligands such as AGEs and pro-inflammatory S100/calgranulins and HMGB1 appear to persist, a situation that facilitates chronic cellular stress.

For example, in the diabetic murine and human kidney, increased expression of AGEs and S100 epitopes in early and advanced stages of kidney disease has been observed in the vascular cells, infiltrating immune cells and target cells such as the podocyte [26]. In an analogous manner, in murine and human macrovascular disease, specifically, atherosclerosis, RAGE ligands co-localize with RAGE expression in macrophages and other immune cells and smooth muscle cells in atherosclerotic plaques [27, 28]. Hence, based on these findings, experiments have been performed in animal models to probe mechanistic links between enhanced RAGE ligands and RAGE expression in the diabetic tissues.

3.1. Accelerated atherosclerosis

Definitive support for mechanistic roles for diabetes in the acceleration of atherosclerosis was published in studies in which atherosclerosis-prone mice either devoid of apolipoprotein E (APOE) or the low density lipoprotein receptor (LDLR) rendered hyperglycemic displayed increased lesion area and complexity compared to their non-diabetic cohorts. Genetic deletion of *Ager* in both diabetic models resulted in reduced atherosclerosis and importantly, macrophage content per lesion area was lower, in parallel with increased lesional collagen, a marker of stability (Figure 1) [29, 30]. The ligands of RAGE also accumulate in non-diabetic atherosclerosis, but to a significantly lesser degree. Even in non-diabetes, roles for RAGE in the pathogenesis of atherosclerosis in murine models was shown [31, 32].

Cell type-specific roles for RAGE in the pathogenesis of atherosclerosis were also shown for bone marrow-derived cells and smooth muscle cells (SMCs) in diabetic mice [30, 33] and in endothelial cells, in non-diabetic mice [32]. Pharmacological approaches to suppressing RAGE-mediated effects in diabetic atherosclerosis were shown with treatment with sRAGE [28], and in uremic mice, with treatment with a neutralizing antibody against RAGE [34]. Further, data in non-diabetic mice revealed that one of the benefits of simvastatin therapy on suppression of atherosclerosis was via downregulation of RAGE and its ligand HMGB1 in the vasculature [35].

Taken together, these considerations suggest that RAGE and its oxidative and inflammatory ligands accumulate in diabetic atherosclerosis and that blockade or genetic deletion of RAGE exerts benefit, without effect on glycemia or levels of total cholesterol and triglyceride. Evidence of roles for the RAGE pathway in non-diabetic atherosclerosis underscore that RAGE and its ligands are also expressed in the inflamed and pro-oxidative environments of non-diabetic atherosclerosis as well. These finding strongly suggest that targeting RAGE might be a complementary therapeutic approach in macrovascular disease, especially in diabetes. In the sections to follow, we consider the effects of RAGE in microvascular complications of diabetes.

3.2. Nephropathy

As discussed above, RAGE is highly expressed in human and murine diabetic kidney. Experiments testing the benefits of pharmacological intervention in models of types 1 and 2 diabetes have reported that administration of sRAGE, alagebrium (AGE cross-link breaker), low molecular weight heparin, or neutralizing anti-RAGE antibodies exerted protection against the effects of diabetes on pathological and functional outcomes in the diabetic kidney [36–40].

In genetically modified models using gain- and loss-of function strategies, RAGE was linked to the pathogenesis of nephropathy [8, 37–39]. Specifically, deletion of *Ager* was shown to prevent mesangial sclerosis, thickening of the glomerular basement membrane, podocyte loss and reduced urinary albumin excretion [8, 37, 38]. As a key endpoint for nephropathy in clinical trials will be the prevention of loss of renal function, studies by Reiniger and colleagues specifically addressed the effects of *Ager* deletion on inulin clearance. Although diabetic OVE26 mice displayed a significant reduction in inulin clearance over a >6 month period, OVE26 mice devoid of *Ager* were protected from the loss of renal function, as measured by inulin clearance [8]. Importantly, in those studies, levels of blood glucose did not differ between OVE26 mice expressing or devoid of *Ager*. Of note, interesting pathogenic roles for bone marrow-derived *Ager* expression in diabetic mice have been shown in bone marrow transplantation studies [41]. However, although diminished podocyte loss and less loss of renal function were observed in the recipients of *Ager*-deficient bone marrow, the degree of glomerulosclerosis and collagen deposition was not affected, thereby suggesting that the more complete protection against indices of nephropathy in diabetes in global *Ager* null mice was only due, in part, to the bone marrow-derived cells.

In recent studies, the effects of pharmacological agents currently in use in diabetic subjects were tested for their effects on the expression of the ligand-RAGE axis in the diabetic kidney. Treatment of diabetic mice with agents such as gemigliptin, an inhibitor of dipeptidyl peptidase 4 (DPP4) [42], empagliflozin (a sodium glucose cotransporter 2 (SGLT2) inhibitor) [43], ramipril (angiotensin converting enzyme inhibitor) [44], and nifedipine, a calcium channel blocker [45], exerted their beneficial effects on nephropathy endpoints, in parallel with reduced expression of the ligand-RAGE axis. Such studies suggest that complementary, multi-targeted treatments, and not single target approaches, may be the most logical means to optimally treat diabetic nephropathy.

3.3. Retinopathy

Data reported from industrialized countries indicate that retinopathy is the most frequent complication of diabetes in the microvasculature and in the working age population, diabetes represents the leading cause of blindness [46]. RAGE and its ligands are expressed in multiple key cell types in the diabetic retina in experimental models, such as in retinal pigment epithelial cells, Muller ganglia and retinal endothelial cells. Exposure of these cells to RAGE ligands induces RAGE-dependent cellular stress [47–49]. Administration of sRAGE or genetic deletion of *Ager* in mouse models of type 1 or type 2 diabetes results in protection against multiple early retinopathic abnormalities in mouse models, including retinal permeability, loss of retinal neuronal function, endothelial and pericyte damage, and microgliosis and inflammatory perturbation [7, 50, 51]. It is acknowledged that one pitfall of rodent models is the limitation that most of the pathology does not represent the advanced stages of disease, such as microaneurysms and retinal hemorrhage.

Given the importance of diabetic retinopathy as a complication of diabetes, it is plausible that therapeutic strategies targeting RAGE might be implemented early in the course of the disease, in order to potentially prevent the earliest vascular and permeability abnormalities. Hence, a focus on local intraocular RAGE-directed therapies as a key strategy may ultimately be most logical to ensure optimal bioavailability and safety.

3.4. Distinct complications of diabetes

In distinct complications of diabetes, such as cardiac abnormalities [52], neuropathy [53] and impaired wound healing [54], as examples, roles for the RAGE signaling pathway have been demonstrated. It is important to note that particularly in full thickness excisional wound healing, topical or intraperitoneal administration of soluble RAGE exerted benefit in enhancing wound closure in type 2 diabetic db/db mice and importantly, in non-diabetic mice, topical treatment with soluble RAGE exerted no adverse effect on the wound healing process, suggesting that the receptor activation was largely triggered in *pathological* dermal damage and not in homeostatic responses to cellular stress [55]. Together with evidence that deletion or blockade of *Ager* generally does not exacerbate or mediate adverse responses to infection, but improves survival in severe sepsis in animal models [56], these considerations suggest that targeting RAGE is not likely to exert deleterious effects in chronic disease conditions in which superimposed acute illnesses may occur. In the section to follow, we review the evidence linking RAGE to obesity and the pathogenesis of insulin resistance and diabetes.

4.0. Obesity and Metabolic Dysfunction and the Growing Links to RAGE

High fat diets in human subjects and/or in animal models generate RAGE ligands such as CML-AGE, phosphoethanolamine modifications of isolevuglandins, HMGB1, and S100B, which accumulate in metabolic tissues in obesity [57–61]. RAGE is expressed to greater degrees in obese versus lean human adipose tissue, particularly in macrophages and adipocytes [59]. When mice globally devoid of *Ager* or their littermate controls were fed a high fat diet (HFD) (60% kcal/by fat) versus a standard low fat diet (LFD), surprisingly, despite equal degrees of food consumption, mice devoid of *Ager* fed HFD gained

significantly less weight versus the littermate *Ager*-expressing controls [60]. In parallel with this observation, mice devoid of *Ager* displayed lower macrophage content in the visceral adipose tissue (VAT) and their adipose tissue macrophages expressed significantly higher levels of “M2”/anti-inflammatory polarized macrophages versus the wild-type controls fed the HFD. Metabolic testing revealed that mice devoid of *Ager* displayed higher glucose and insulin tolerance and by studies in metabolic cages, higher energy expenditure [60]. Consistent with partial yet significant roles for myeloid-derived RAGE in these phenomena, wild-type mice subjected to lethal irradiation and bone marrow transplantation with *Ager* null versus wild-type bone marrow displayed significantly less weight gain, metabolic dysfunction, and macrophage content with increased “M2” like inflammatory polarization compared to mice reconstituted with *Ager*-expressing bone marrow [60].

To ensure that these findings were not due to innate roles for RAGE in metabolic organ development, we treated adult wild-type mice with sRAGE or vehicle, beginning at the time of, or three weeks after the initiation of HFD. In both time courses, compared to vehicle-treated mice, mice treated with sRAGE displayed significantly slower weight gain despite equal degrees of food consumption [60]. Further studies are underway to identify the precise cellular depots and mechanisms that account for these roles for RAGE in obesity and high fat feeding. Therefore, this work suggests that chronic treatment with RAGE antagonists may not result in untoward weight gain and consequent metabolic dysfunction. This concept requires definitive testing in human subjects.

5.0. RAGE and Roles in Autoimmunity and Chronic Inflammation

The discovery that RAGE was a receptor for non-AGE ligands such as S100/calgranulins, HMGB1 and Mac1, as examples [9, 10], set the stage for delineating roles for this receptor in non-diabetic inflammation. The discovery of RAGE expression and function in T lymphocytes underscored that RAGE played roles in T cell activation and differentiation and first linked RAGE to the pathogenesis of autoimmune type 1 diabetes [62, 63]. Hence, analogous to the studies discussed above in obesity, this work of Herold and Clynes showed that RAGE contributed to the pathogenesis of type 1 diabetes.

In human subjects, a particular polymorphism of RAGE (“RAGEG82S”) was found to be in linkage disequilibrium with HLA-DR4, which is classically associated with rheumatoid arthritis [64, 65]. Mice vulnerable to autoimmune bone and joint tissue destruction treated with sRAGE displayed significantly less joint inflammation and bone loss compared to mice treated with the vehicle. Cultured cells expressing G82S versus the G82G wild-type displayed higher binding affinity for S100 ligands and greater degrees of matrix metalloproteinase-mediated inflammation upon treatment with the RAGE ligand S100B [64]. RAGE has been shown to be expressed in other forms of autoimmune disease, such as in the minor salivary glands of patients with Sjogrens’ syndrome [66].

It was reported that phosphatidylserine (PS) is a RAGE ligand; PS, when translocated from the inner to the outer plasma membrane, serves a signal for apoptotic cell clearance. It was shown that mice devoid of *Ager* subjected to inhalation of lipopolysaccharide (LPS) displayed reduced clearance of apoptotic neutrophils versus the wild-type *Ager*-expressing

mice [14]. Impaired clearance of apoptotic cells is also a hallmark of systemic lupus erythematosus, which is modeled in B6-MRL Fas lpr/j mice; time course experiments of annexin V expression revealed that autoreactive T lymphocytes in the spleen of B6-MRL Fas lpr/j mice devoid of *Ager* exhibited delayed apoptosis and expressed significantly less activated caspase 3 than T lymphocytes in B6-MRL Fas lpr/j mice expressing RAGE. These studies suggested that RAGE reduces the accumulation of autoreactive CD3(+)B220(+)CD4(-)CD8(-) T lymphocytes, and that deletion of *Ager* exacerbates lymphoproliferative syndrome, autoimmunity, and organ injury. From the studies, we may infer that RAGE is able to rescue apoptosis of T lymphocytes when Fas/CD95 is not present or functional [67].

One consequence of chronic inflammation in diseases such as SLE is accelerated atherosclerosis [68]; given the upregulation of RAGE ligands and early endothelial stress and dysfunction that marks both disorders, it is not surprising that the RAGE pathway was linked to cardiovascular diseases in autoimmune settings [69].

Finally, in the context of RAGE-dependent mechanisms in inflammation and autoimmunity, it is established that the proinflammatory ligand HMGB1 binds both RAGE and to toll-like receptors (TLRs); the latter are key gate keepers of innate immunity. To begin to address the inter-relationship and/or inter-dependence of RAGE versus TLR signaling, we employed extensive liver resection (85%) as an *in vivo* model system for testing this concept. Although Myd88, downstream of certain of the toll-like receptors, is required for survival via regulation of NF- κ B and TNF- α , deletion of *Ager* was associated with significantly higher survival compared to wild-type, *Myd88* null, or *Ager/Myd88* null mice (Figure 2). Studies performed in the hepatic remnant indicate that RAGE opposes Myd88 signaling by the following mechanisms: (1) RAGE suppresses NF- κ B p65 and consequent production of cyclin D1; and (2) RAGE suppresses Pim1 through a STAT3/Il6-mediated mechanism, which will both increase apoptosis and reduce the hyperplastic response [70]. From these studies, we deduced that toll-like receptor signaling was essential for activation of innate mechanisms after massive injury that were required for the animal to survive the acute stress of massive resection of the liver, and that RAGE action blunted the benefits of toll like receptor actions through inhibition of survival mechanisms in the liver, including NF- κ B and Pim1. Taken together, these reports suggest that RAGE action is largely deleterious in acute stresses and therefore support the premise that extended blockade of RAGE in long-standing chronic disease may not adversely block adaptive pathways needed for acute survival in immediate stress responses.

6.0. RAGE and Cancer – Many Ligands Playing Key Roles in Tumor Behavior

The discovery of HMGB1 as a RAGE ligand set the stage for uncovering if RAGE played roles in cellular migration, a key component of local tumor growth and metastases. In our first study, we showed that administration of sRAGE or antibodies to RAGE was highly protective against local tumor growth and invasion in C6 glioma-raised tumors and in distant tumor metastases in the Lewis lung carcinoma model [10]. Since that discovery, studies in

human subjects have uncovered that RAGE is expressed in a broad array of human tumors, such as cancers of the breast, gastric system, colon, liver and hepatobiliary system, pancreas, prostate, oral cavity (squamous cell cancer), lung, brain and skin (melanoma) [71]. The HMGB1-RAGE axis has recently been shown through bioinformatics approaches to be a strong predictor of poor prognosis in lung cancer [72]. Beyond HMGB1, multiple other ligands of RAGE have since been implicated in tumor biology, including S100A4, S100P, S100A12, S100A7, S100A8/9, S100B, S100A14, S100A2, CML-AGE, and lysophosphatidic acid [12, 73–87].

Multiple mechanisms by which the ligand-RAGE axis exerts its effects in tumors have been described, including facilitating angiogenesis and endothelial cell proliferation; tumor cell invasion, migration and metastasis; modulation of the tumor microenvironment; promotion of autophagy; actin-polymerization; epithelial-mesenchymal transition; chemoattraction of myeloid derived macrophages; upregulation of inflammatory signaling; and protection against apoptosis [74, 75, 77, 80–82, 84–88].

RAGE is highly expressed in macrophages and emerging data suggest that RAGE regulates macrophage content and inflammatory polarization in diabetic atherosclerosis, diabetic sciatic nerve injury and in adipose tissue macrophages in high fat feeding [30, 60, 89]. Roles for RAGE in immune cells in tumors, in particular, tumor-associated macrophages (TAMs) were definitively suggested by bone marrow chimera studies in mice subjected to an inflammation-induced skin tumor; in this setting, mice globally devoid of *Ager* were resistant to the development of the skin tumor [90]. The site of RAGE-dependent action was identified to be the bone marrow, based on bone marrow transplantation studies in mice. In other studies, it was shown that RAGE mediates ligand S100A7-mediated breast cancer growth through recruitment of MMP9-expression in TAMs [77]. In the setting of glioma tumors, RAGE expression in TAMs contributes to the promotion of tumor-associated inflammation and angiogenesis [91], a process mediated potentially by the ligand S100B [82].

Finally, additional RAGE-dependent mechanisms in tumors may include the effect of this receptor on regulation of transcription and activation of *Egr1* in hypoxia, thereby increasing conditions favorable for tumor growth and spread. In hypoxic endothelial cells, cardiomyocytes, and macrophages [92–94], deletion of *Ager* blocked hypoxia-mediated upregulation of *Egr1*; EGR1 plays major roles in regulation of inflammatory and prothrombotic genes in the hypoxic state [95]. In other studies, RAGE was linked to the pathogenesis of hypoxia induced stress in cystic fibrosis models in mice [96] and through down-regulation of *Ager* by silencing of hypoxia inducible factor 1 (HIF-1a) in breast tumor samples [97].

Taken together, there are multiple putative mechanisms by which RAGE may modulate tumor biology, that is, through direct effects on tumor cell proliferation, survival, migration and invasiveness; through maladaptive modulation of the tumor environment, particularly in TAMs; through enhancement of angiogenesis through effects on endothelial cells; and through upregulation of damaging proinflammatory and prothrombotic transcription factors in hypoxia, a common complication that occurs in the tumor milieu.

7.0. RAGE in the Lung: Possible Physiological Roles

In the absence of disease, low levels of RAGE are expressed in most body tissues, including vascular tissues, immune cells, neurons, cardiomyocytes, podocytes and certain epithelial cells, as examples. In contrast, the expression of RAGE is naturally high in the lung in homeostasis [98]. In the normal lung, RAGE is largely expressed in alveolar type I pneumocyte (ATI cell) [99, 100], where it is localized to the basolateral membrane of these cells, suggesting possible roles in mediating the contact between ATI cells and their natural substratum [100, 101]. Interestingly, in lung cancer, expression levels of RAGE are reported to be reduced compared to those of the non-tumor adjacent tissue [102]. Such reduction in levels of RAGE in lung cancer was suggested to support the growth of lung cancers, such as non-small cell lung tumors [103].

In mouse models of lung stress, induction of silicosis resulted in down-regulation of RAGE expression and, in mice devoid of *Ager*, a generally higher histological score (*i.e.*, greater fibrotic pathology) compared to wild-type control mice [104]. In the setting of intratracheal treatment of mice with bleomycin, however, although mice devoid of *Ager* displayed the same early inflammatory infiltrates as wild-type mice after treatment, the mice devoid of *Ager* displayed higher overall survival and less lung fibrosis compared to the *Ager*-expressing controls [105]. Upon intratracheal administration of lipopolysaccharide, mice devoid of *Ager* were not protected from the ill effects when compared to the wild-type mice; however, upon intratracheal administration of *E. coli*, mice devoid of *Ager* displayed less inflammation, lower expression of inflammatory mediators, and overall less evidence of pneumonia [106]. Taken together, these considerations suggest that the function of RAGE in the lung is complex and that further experimentation is required to determine if blockade of the RAGE pathway would induce any negative outcomes in this organ.

8.0. Alzheimer's Disease and Neurodegeneration

One of the ligands of RAGE is the amyloid-beta peptide (A β) and β -sheet fibrils [11]; these species accumulate in Alzheimer's disease brain and are believed to play key roles in the pathogenesis of the disease. In addition to A β , other ligands of RAGE have been linked to AD, such as AGEs and HMGB1 [107–109]. It has been suggested that RAGE ligands stimulate beta secretase (BACE1), a mechanism to increase levels of A β [108, 109]. Further, it has been shown that diabetes exacerbates amyloid plaque deposition in AD transgenic mouse models, at least in part through AGE/RAGE-mediated activation of NF- κ B [110]. In human subjects, RAGE is expressed in AD brain, especially in the hippocampus [111] and interestingly, the RAGE G82S polymorphism was suggested to be involved in increased genetic susceptibility to AD, albeit in a small preliminary study [112].

RAGE is expressed in many of the cell types linked to the pathogenesis of AD. As illustrated in Table 2, experiments using cultured cells, primary cells, cell-type specific transgenic and genetically-modified mice implicated roles for RAGE in microglia, astrocytes, neurons and in the vasculature. Indeed, one possible mechanism by which RAGE contributes to AD is in opposing roles and alterations in A β transport with LRP-1 (low density lipoprotein receptor related protein 1) [113–117].

The possibilities for therapeutic opportunities targeting RAGE in AD are mounting; in addition to agents being tested against RAGE in preclinical models [118], a inhibitor of RAGE is in clinical trials after evidence of potential efficacy in prevention in cognitive decline in human subjects with Alzheimer's Disease [119, 120].

9.0. RAGE Signaling – the Discovery of the Formin DIAPH1 as an Intracellular Signaling Effector of RAGE

The cytoplasmic domain of RAGE is short and highly charged and is essential for RAGE ligand-stimulated signaling. To discover the molecular mechanisms by which RAGE activates signaling pathways, we used a yeast-two-hybrid assay to identify binding partners of RAGE. Through this approach, we uncovered multiple copies of the formin molecule, DIAPH1 [121]. Formins are cytoplasmic actin-binding proteins that play key roles in cellular migration and actin cytoskeleton dynamics; as effectors of the Rho GTPases (RhoA, Rac1, Cdc42) involved in multiple components of intracellular signaling; and as regulators of Serum Response Factor (SRF) transcription factor and its downstream genes [122, 123].

Beyond evidence of the interaction of the RAGE cytoplasmic domain with DIAPH1 by co-localization studies in cellular models and by co-immunoprecipitation and Western blotting from cellular lysates, it was essential to test if DIAPH1 was required for RAGE signaling. To date, in primary murine SMCs, cultured BV2 microglia, human thyroid cancer cells and murine and human macrophages and THP1 cells, small interference RNA-mediated knockdown of *Diaph1* or deletion of *Diaph1* resulted in significantly reduced signaling in response to RAGE ligands such as S100B, CML-AGE, S100A4 and hypoxia-derived AGEs [73, 92, 124, 125], particularly manifested as reduced activation of Rac1, RhoA and cdc42. *In vivo*, neointimal expansion in the femoral artery consequent to guide wire induced injury was significantly lower in mice devoid of *Diaph1* versus the wild-type control; key roles for RAGE/DIAPH1 signaling in SMCs accounted for these effects [125]. Of note, the response noted in mice devoid of *Diaph1* was highly analogous to that observed in mice devoid of *Ager*, suggesting a common mechanistic link [126].

Recently, Shekhtman and colleagues used NMR spectroscopy to identify the four key amino acids in the RAGE cytoplasmic domain (Q3, R4, R5 and Q6 corresponding to Q364, R365, R366, and Q367 of the full-length RAGE) that were essential to interact with the FH1 domain of DIAPH1 (Figure 3) [127]. When R5/Q6 were mutated to alanine residues and expressed in murine SMCs, Akt signaling and cellular migration and proliferation were significantly reduced, indicating that these residues in the RAGE cytoplasmic domain were required for RAGE signaling [127].

In a recent study, it was suggested that the RAGE cytoplasmic domain may interact with DOCK7 to regulate activation of cdc42 signaling [128]; it will be important to test if DOCK7 is part of a multimolecular component unit, perhaps including DIAPH1, that is required to fully activate Rho GTPase signaling initiated by RAGE ligands.

10.0. Expert Opinion

The unexpected finding that mice devoid of *Ager* were protected from HFD-induced obesity placed RAGE squarely in the midst of metabolic dysfunction [60]. In fact, it was surprising to discover that HFD stimulated increases in multiple families of RAGE ligands in metabolic organs, such as CML-AGEs, HMGB1 and S100/calgranulins, even in the absence of frank hyperglycemia [57–61]. These considerations thus suggested that RAGE was not only involved in the pathogenesis of diabetic complications, but also in the biochemical and metabolic perturbations that characterize the pre-diabetic or diabetic-susceptible state. These fascinating concepts set the stage for uncovering the “natural function” of RAGE and for understanding the breadth of its links to metabolic regulation. It is thus tempting to speculate that antagonism of RAGE in clinical trials might yield benefits beyond preventing or assuaging inflammation-induced exacerbation of tissue damage in diabetes, specifically by limiting weight gain and metabolic abnormalities even in subjects consuming unhealthy diet and with limited physical activity. Furthermore, as discussed above, the intriguing biology of RAGE in the lung has yet to be fully deciphered. No doubt, however, that all induced lung pathologies in the *Ager* null mouse are not strictly associated with negative outcomes, such as in the setting of bleomycin-induced fibrosis, in which mice devoid of *Ager* display protection, not greater disease. Experiments in tissue-targeted *Ager*-modified mice are likely to be helpful in settling this quandary.

The body of work accrued to date on the biology of RAGE also underscores that the RAGE pathway and its links to disease do not represent “one ligand-one disease.” Indeed, beyond obesity and diabetes, as illustrated above, similar considerations were discussed in this review with respect to other RAGE-associated diseases, such as autoimmunity, Alzheimer’s disease, and cancers, that is, more than one class of RAGE ligands is upregulated and accumulated in specific chronic disease settings.

Is one class of ligand more important than another in terms of disease pathogenesis? Given the expanding number of RAGE ligands and the fact that not all may be approached genetically by deletion, for example, for strategic reasons and hypothesis testing, we surmise that creative solutions to targeting RAGE will be needed. Indeed, the solution structure of the RAGE extracellular domain suggests that the ligand-binding domain is an elongated molecule containing two “patches” that mediate the interaction with ligands – a large basic patch and a large hydrophobic patch. Interestingly, both are highly conserved, suggesting the evolutionary importance and relevance, perhaps, of RAGE in chronic inflammation. Importantly, whereas S100B is largely recognized by the hydrophobic patch in the extracellular domain (residues 54–67), AGEs, in contrast, mediate their interaction with RAGE via their negative charges [129–131].

Importantly, studies have suggested that RAGE may contribute to regulation of its ligand levels. As illustrated in Figure 4, RAGE-dependent downregulation of GLO1 may limit the detoxification of the pre-AGEs, thereby increasing levels of AGEs. Further, if validated, RAGE-dependent levels of BACE1 may have implications for the sustained generation of A β in settings of high RAGE expression. The potential utility of BACE1 inhibitors as a viable therapeutic strategy in AD has yet to be definitively established [132]. It is also

conceivable that GLO1 stimulating agents and BACE1 inhibitors might be important adjunctive strategies when coupled with RAGE antagonism.

Where, then, does this leave reasonable prospects for anti-RAGE therapeutic development? The first agent tested in antagonism of the RAGE pathway was sRAGE. Administration of sRAGE to rodents was remarkably protective against the consequences of diabetes, high fat feeding, inflammation and tumors [1–4]. There are challenges to the development of sRAGE as a viable therapeutic agent, however. sRAGE, presumably acting as a ligand sink to sequester RAGE ligands and prevent their interaction with the cell surface receptor, would require long-term administration in chronic diseases. Although surmountable, the costs of production and delivery might not be inconsequential. More importantly, on a biological basis, is it possible that sRAGE might block the interaction of ligands with beneficial receptor pathways? Just as the receptor is promiscuous with respect to its diverse ligand repertoire, the ligands do not display fidelity to a single receptor, either. Although no evidence of maladaptive effects emerged from long-term sRAGE treatment in mice, it is the case that the mice were not challenged with superimposed stresses, such as infection, immunization, physical wounding or other stimuli. Would sequestering RAGE ligands from their receptor(s) exert negative consequences? We do not have the entire answer to this question at this time.

Although sRAGE is present in the circulation of human subjects, the extent to which it may be functional, that is, that it may possess the ability to effectively sequester maladaptive (or adaptive) ligands, is not established. Indeed, at this time, the study of sRAGE in human subjects is focused on the potential of this material to serve as a biomarker for chronic illness. Even in that setting, studies on measurement of soluble RAGEs in human subjects and their relationship to human health and disease further underscore that the biology of soluble RAGE is complex. For example, in cardiovascular disease (CVD), lower levels of sRAGE were reported to align with both worse CVD [133] and the presence of essential hypertension compared to normal age-matched control subjects [134]. However, measures of sRAGE in subjects with diabetes revealed that higher levels of sRAGE were associated with greater degrees of CVD [135].

In the setting of lung disease, high levels of sRAGE were reported to be predictors of negative outcome in lung transplantation [136]. Yet, in a distinct study, lower levels of sRAGE were associated with the degree of severity of emphysema [137].

Taken together, there is, to date, no consensus on the predictive value of sRAGE as a biomarker in disease vs. health, and disease status and extent (stage). We speculate that perhaps sRAGE measures might be of greater predictive value were each subject, prior to natural progression or disease treatment, to serve as “their own control,” such as recently published in subjects undergoing bariatric surgery [138]. In that study, the baseline level of sRAGE in the individual subject predicted the overall metabolic response, or not, to bariatric surgery.

Thus, beyond sRAGE, distinct strategies to target the extracellular domains of RAGE such as small molecule antagonists, anti-RAGE antibodies and RAGE peptide aptamers or full-

length RAGE by using nanocarriers carrying RAGE siRNAs, have been tested in animals and in few cases, in human subjects to date [118–120, 139, 140]. We propose that a logical means to target the RAGE pathway may be by interfering with its signaling. As noted above, to date, distinct classes of ligands have been shown to signal through DIAPH1, including AGEs and different members of the S100/calgranulin family. Certainly, more research is required to understand the entire scope of RAGE signaling and the extent to which blocking RAGE/DIAPH1 interaction may encompass the full pathobiology of RAGE activation. Small molecule inhibitors of this pathway, under active development at this time, may serve as novel probes for dissecting RAGE signaling in response to distinct ligands, as well as templates for therapeutic development. Given the potent effects of RAGE activity in animal models and its ability to be tracked *in vivo* by measures of soluble RAGEs and RAGE levels in accessible peripheral blood-derived immune cells, the time appears to have come to rigorously interrogate the RAGE pathway as a logical therapeutic target for chronic disease.

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Reference annotations

* Of interest

** Of considerable interest

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Article Highlights Box

- RAGE is a multi-ligand receptor of the immunoglobulin superfamily; RAGE is expressed on multiple cell types such as vascular and immune cells, as well as target cells in diabetic complications. The expression of RAGE in most organs is low in homeostasis; a notable example is the lung, in which high levels of RAGE are observed in health.
- Pharmacological modulation of, or genetic deletion of *Ager*, is protective in aging, in multiple animal models of diabetic complications, in obesity and metabolic dysfunction, in autoimmune and inflammatory settings, in tumors and in models of Alzheimer's disease.
- The cytoplasmic domain of RAGE binds to the formin DIAPH1. Knockdown of *Diaph1* or genetic deletion of *Diaph1* blocks RAGE signaling in cell types such as smooth muscle cells, macrophages, microglial cells and transformed cells.
- The critical amino acids in the RAGE cytoplasmic domain have been identified that underlie the interaction between this RAGE domain and the FH1 domain of DIAPH1. Mutation of these amino acids in the RAGE cytoplasmic domain blocks the interaction with DIAPH1 and blocks RAGE ligand-induced signal transduction.
- The finding that mice devoid of *Ager* are viable and fertile; they display protected responses to many forms of infection, sepsis and massive liver injury (resection); and they have normal life span, strongly suggests that blocking RAGE signaling is not likely to cause deleterious side effects. Hence, targeting the cytoplasmic domain of RAGE-DIAPH1 interaction with small molecule inhibitors holds great potential to safely block RAGE signaling and prevent the consequences of chronic disease. Definitive testing in human subjects is required.

Figure 1. Deletion of *Ager* suppresses diabetes-accelerated atherosclerosis and effect of diabetes and *Ager* deficiency on macrophage and smooth muscle cell lesional content

(a). Male *ApoE* null and *ApoE* null/*Ager* null mice were rendered diabetic with streptozotocin at age 6 weeks. Mice were sacrificed and aortas were retrieved. Mean atherosclerotic lesion area at the aortic sinus is shown. (b). Immunostaining and Picrosirius Red staining of atherosclerotic lesions from the indicated *ApoE* null mice was performed for detection of macrophages, smooth muscle cells, T cells and collagen per lesion area (the latter using picrosirius red and polarizing microscopy) at age 24 weeks. Statistical considerations: * indicates $p < 0.03$ vs. *ApoE* null/*Ager* null diabetic mice; ** indicates $p < 0.01$ vs. *ApoE* null/*Ager* null diabetic mice; and ^ indicates $p < 0.02$ vs. *ApoE* null/*Ager* null non-diabetic mice. Adapted from *Circ Res* 2010;106:1040–1051.

Figure 2. Effect of *Ager* or/and *Myd88* gene deletion on survival after extensive (85%) hepatectomy

Kaplan-Meier curves for Wild-type (WT), *Ager* null, *Myd88* null, or *Myd88/Ager* null mice were plotted, and the statistical significance of the probability of survival of the mutants vs. WT mice was calculated. $n = 10$ mice/group. Statistical considerations: *Ager*^{-/-} vs. WT, $P = 0.01$; *Ager*^{-/-} vs. *Myd88*^{-/-}, $P < 0.001$; *Myd88*^{-/-} vs. WT, $P = 0.013$; *Myd88*^{-/-} vs. *Myd88*^{-/-}/*Ager*^{-/-}, $P = 0.144$. Adapted from *FASEB J* 2012; 26: 882–893.

Figure 3. Cytoplasmic tail (ct)RAGE interacts with DIAPH1 FH1 domain

(a). Sequence alignment of the human DIAPH1 FH1 construct used in this study (NCBI accession code [NP_005210](#)) and mouse DIAPH1 FH1 (NCBI accession code [NP_031884](#)). Conserved residues are in *red*. (b). Overlay of ^{15}N -edited heteronuclear single quantum coherence, HSQC, NMR spectra of free [$U\text{-}^{15}\text{N}$] ctRAGE (*black*) and the DIAPH1 FH1- [$U\text{-}^{15}\text{N}$]ctRAGE complex (*red*). To form the DIAPH1 FH1-ctRAGE complex, 0.5 mM unlabeled DIAPH1 FH1, in NMR buffer (10 mM potassium phosphate (pH 6.5), 100 mM NaCl, 0.02% (w/v) NaN_3 , in 90%/10% $\text{H}_2\text{O}/\text{D}_2\text{O}$) was added into 100 μM [$U\text{-}^{15}\text{N}$]ctRAGE to yield a DIAPH1 FH1/ctRAGE molar ratio of 1:1. Due to ^{15}N editing of the experiment, only backbone and side chain amide protons and nitrogens of ctRAGE are present in the spectrum. Most peaks do not change their positions, reflecting the fact that only a subset of ctRAGE residues interact with FH1. ctRAGE peaks that are substantially or completely broadened are labeled. Note that Q3, R4, R5, and Q6 of ctRAGE correspond to Q364, R365, R366 and Q367 of the full length RAGE (c). DIAPH1 FH1-ctRAGE interaction map. Residues broadened during the NMR titration experiment are indicated in *red*. *J Biol Chem* 2012; 287:5133–5144.

Figure 4. RAGE & regulation of ligand levels

Content of two of the classes of RAGE ligands, glyoxal (G) and methylglyoxal (MG)-derived AGEs, and amyloid- β -peptide ($A\beta$), may be regulated, in part, by RAGE, in chronic disease settings. *First*, in the case of AGEs, the key pre-AGE intermediates, G and MG, are detoxified by the enzyme glyoxalase 1 (GLO1). Published evidence indicates that RAGE downregulates *Glo1* mRNA and activity in certain diabetic tissues. Hence, RAGE activation in high-AGE settings may stimulate AGE production and accumulation via a feed-forward loop in which RAGE suppresses the brake on pre-AGE (G & MG) detoxification, thereby favoring more conversion into AGEs. These considerations have implications for conditions such as diabetes complications. *Second*, in the case of $A\beta$, published evidence suggests that ligand-RAGE interaction might upregulate BACE1, thereby promoting further production of $A\beta$. These considerations have implications for conditions such as Alzheimer's disease. *Abbreviations:* AGE, advanced glycation endproduct; G, glyoxal; GLO1, glyoxalase 1; and MG, methylglyoxal.

Table 1

Examples of RAGE ligands *

Ligand	Reference
AGEs	[4, 5]
Amyloid β -peptide	[11]
HMGB1	[10]
Lysophosphatidic acid	[12]
Mac-1	[13]
Phosphatidylserine	[14]
S100/calgranulins	[9]

* Note that other putative ligands of RAGE have been described; this list represents the more commonly described ligands of the receptor

Table 2

CNS Cell Types Implicated in AD via RAGE-dependent mechanisms *

Ligand	Reference
Astrocytes	[141, 142]
Endothelial	[114, 115, 142]
Microglia	[113, 117]
Neurons	[11, 116]

* In this Table, we refer to the more commonly studied cell types implicated in AD; this does not exclude the potential involvement of other RAGE-expressing cell in the CNS in the pathogenesis of AD.