The RAGE Axis
A Fundamental Mechanism Signaling Danger to the Vulnerable Vasculature

Shi Fang Yan, Ravichandran Ramasamy, Ann Marie Schmidt

Abstract: The immunoglobulin superfamily molecule RAGE (receptor for advanced glycation end product) transduces the effects of multiple ligands, including AGEs (advanced glycation end products), advanced oxidation protein products, S100/calgranulins, high-mobility group box-1, amyloid-β peptide, and β-sheet fibrils. In diabetes, hyperglycemia likely stimulates the initial burst of production of ligands that interact with RAGE and activate signaling mechanisms. Consequently, increased generation of proinflammatory and prothrombotic molecules and reactive oxygen species trigger further cycles of oxidative stress via RAGE, thus setting the stage for augmented damage to diabetic tissues in the face of further insults. Many of the ligand families of RAGE have been identified in atherosclerotic plaques and in the infarcted heart. Together with increased expression of RAGE in diabetic settings, we propose that release and accumulation of RAGE ligands contribute to exaggerated cellular damage. Stopping the vicious cycle of AGE-RAGE and RAGE axis signaling in the vulnerable heart and great vessels may be essential in controlling and preventing the consequences of diabetes. (Circ Res. 2010;106:842-853.)

Key Words: diabetes ■ cardiovascular complications ■ RAGE ■ inflammation

The multiligand receptor for advanced glycation end products (RAGEs) is a signal transduction receptor of the immunoglobulin superfamily. The ligand families of RAGE are implicated in fundamental acute and chronic stress responses (Table). Although first described as a receptor for the advanced glycation end products of nonenzymatic glycation and oxidation of proteins (AGEs), RAGE also transduces signals stimulated by molecules released and/or upregulated in inflammatory responses. These non-AGE ligands of RAGE include members of the S100/calgranulin family, high mobility group box (HMGB)-1, and Mac-1. Furthermore, RAGE is a receptor for amyloid-β peptide and β-sheet fibrils characteristic of amyloid.

Extensive epidemiological data indicate that diabetes greatly advances the risk, degree, and consequences of atherosclerosis in the vasculature. In female subjects, the protective effects of hormones in the premenopausal state vanish in the face of hyperglycemia. Thus, elevated levels of...
glucose and their complications may override the benefits of otherwise highly protective gene programs.

Why do hyperglycemic subjects demonstrate amplified tissue damage in diabetic atherosclerosis and in hypoxic and ischemic stresses? The specific importance of glucose itself may somewhat differ in types 1 and 2 diabetes, but it may be too soon to make such a conclusion. Although strict glycemic control was shown to be highly protective in type 1 diabetes and microvascular complications, the benefits of this intervention on macrovascular complications were not uncovered until well beyond the original Diabetes Control and Complications Trial (DCCT) but into the Epidemiology of Diabetes Interventions and Complications (EDIC) study. Thus, in type 2 diabetes, although very recent studies have confirmed that strict glycemic control is beneficial in reducing microvascular disease, the evidence for macrovascular complications is not apparent. Perhaps, as Skyler et al point out, the benefits (as in type 1 diabetes) may not be evident during the course of the Action in Diabetes and Vascular Disease—Preterax and Diamicron Modified Release Controlled Evaluation (ADVANCE) and Veterans Affairs Diabetes Trial (VADT) trials, but rather they may emerge at later times. It is also possible that strict glycemic control in type 2 diabetic subjects had distinct untoward effects, such as weight gain. In fact, the Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial was required to halt the strict glycemic control group because of untoward mortality in that group. Thus, it is possible that in aged subjects, hypoglycemic episodes themselves were more apt to lead to fatal events.

Yet, there are other possibilities as well. No doubt, the type 2 diabetic environment is more likely to be encumbered by various confounding factors, such as hyperlipidemia, obesity, and accompanying insulin resistance, hypertension, and “aging” of the vasculature compared to type 1 diabetes. In this review, we present evidence to support the hypothesis that the RAGE axis is a fundamental contributing mechanism to diabetes and its diverse complications. We propose that although hyperglycemia ignites the generation of AGEs and initial recruitment of RAGE action in the diabetic vasculature, the interplay of RAGE with distinct environmental factors amplifies oxidative stress, vascular inflammation, and mechanisms that compound the risk of macrovascular disease in diabetes.

Table. Ligand Families of RAGE

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Ligand Family</th>
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<tr>
<td>CML-AGE</td>
<td>AGEs</td>
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<tr>
<td>Priony glycinne</td>
<td>AGEs</td>
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<tr>
<td>oxLDL-containing AGE epitopes</td>
<td>AGEs</td>
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<tr>
<td>Advanced oxidation protein products</td>
<td>AOPPs</td>
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<tr>
<td>S100A12</td>
<td>S100/calgranulins</td>
<td>38</td>
</tr>
<tr>
<td>S100B</td>
<td>S100/calgranulins</td>
<td>38</td>
</tr>
<tr>
<td>HMGB1</td>
<td>HMGB1</td>
<td>37</td>
</tr>
<tr>
<td>Amyloid-β peptide</td>
<td>Amyloid/beta sheet fibrils</td>
<td>1, 2</td>
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This table illustrates representative members of the RAGE ligand families.

Posttranslational Modifications and Generation of RAGE Ligands: Coming Face to Face With RAGE

Advanced Glycation End Products

RAGE was born from the discovery that proteins bathed in high glucose were vulnerable to posttranslational modifications and that such glucose-modified proteins, the advanced glycation end products (AGEs), were not mere bystanders in the diabetic microenvironment, but rather active participants in stimulation of signal transduction and target cell dysfunction. AGEs produced in vitro via incubation with reducing sugars and those derived from in vivo sources stimulate cells implicated in diabetic vascular consequences such as endothelial cells (ECs), smooth muscle cells, and monocytes/macrophages. Via activation of signal transduction cascades and transcription factors such as nuclear factor (NF)-κB, AGE-RAGE interaction yields oxidative stress and increased expression of inflammatory and prothrombotic species in atherosclerosis-prone vessels. These factors, coupled with proatherogenic properties of modified low density lipoproteins (LDLs) likely synergize, particularly in diabetes, to accelerate atherosclerosis.

Interestingly, oxidized LDL (oxLDL) is vulnerable to glycoxidation reportedly both on protein and lipid species. We recently showed that oxLDL stimulation of wild-type primary murine aortic ECs increases expression of vascular cell adhesion molecule-1 and matrix metalloproteinase (MMP)-2 protein and activity. Western blotting of oxLDL with anti-AGE IgG antibodies demonstrated strong AGE epitope immunoreactivity in oxLDL but not native LDL species. Furthermore, pretreatment of ECs from wild-type
mice aorta with anti-AGE antibodies greatly suppressed oxLDL-mediated regulation of MMP-2. That RAGE transduced oxLDL-mediated signals was evident by experiments in which incubation of RAGE-deficient murine aortic ECs with oxLDL failed to upregulate inflammatory molecules.11

AGEs are heterogeneous species; hence, a key experimental goal has been to identify the specific AGEs that bind RAGE. In addition to carboxy methyl lysine AGEs, a food-derived AGE, pronyl glycine, may also represent a class of RAGE ligands.12,13 Thus, in addition to innate forces that propel AGE formation such as hyperglycemia and oxidative stress, diets high in AGE content may contribute to the production and accumulation of pathogenic species and vascular damage.

In addition to glucose-, reactive oxygen species (ROS), and food-derived sources of AGEs, hypoxia may generate AGE immunoreactive species.14 In testing the hypothesis that RAGE-dependent ischemia/reperfusion injury resulted, at least in part via regulation of early growth response (Egr)-1, we subjected wild-type and RAGE-null mice to global hypoxia and found that compared to wild-type mice, in which a highly significant increase in Egr-1 mRNA transcripts and nuclear protein and activity resulted in the heart, RAGE-deficient mice subjected to the same degree of hypoxia demonstrated only a small increase in Egr-1 versus the wild-type cohorts. Immunohistochemistry of heart tissue after hypoxia revealed increased expression of Egr-1 in endothelial cells.14 What was the ligand generated in hypoxia that engaged RAGE in this setting?

To address this question, primary murine aortic endothelial cells were retrieved from wild-type mice and subjected to in vitro–applied hypoxia. Although, after multiple rounds of supernatant concentration, neither S100/calgranulin nor HMGB1 epitopes were found, a rapid increase in AGE epitopes, as detected by ELISA using an antibody recognizing heterogeneous AGEs, was demonstrated.14 In wild-type but not RAGE-deficient endothelial cells, hypoxia induced highly significant increases in Egr-1 mRNA and nuclear protein, at least in part via protein kinase C-βII–c-Jun N-terminal kinase (JNK) mitogen-activated protein (MAP) kinase signaling. Consistent with AGEs as mediators of RAGE-dependent regulation of Egr-1 in hypoxia, pretreatment of wild-type mice with an AGE inhibitor (aminoguanidine) or incubation of wild-type endothelial cells with anti-AGE IgG (as well as sRAGE or anti-RAGE IgG) prevented hypoxia-stimulated increases in Egr-1.14

AGEs form in natural aging as well, even in the absence of diabetes. Our first studies have illustrated that the polyol pathway enzyme aldose reductase levels and activity, AGE levels and RAGE expression increase in aging Fischer 344 rat material from which labeled AGE-BSA retrieved RAGE as a binding partner.1 The discovery that S100A12 and amphoterin (HMGB1) bound RAGE initially presented a quandary for several reasons: first, S100s and amphoterin were largely recognized as intracellular molecules.1 S100s were located in the intracellular space wherein at least in part through their “EF-hand” domains, these molecules mediated calcium signaling, phagocytosis and cellular migration.26 Amphoterin was described initially as a nonhistone DNA binding protein located in the nucleus.29 Second, the key signatures of S100s

Advanced Oxidation Protein Products

Another specific consequence of AGE-RAGE interaction is the generation of ROS, at least in part through NADPH oxidase.17 Atop basal modulation of gene expression in high glucose, ROS, such as those driven by the myeloperoxidase pathway, may prompt further AGE ligand formation.18,19 It has recently been shown that “advanced oxidation protein products” (AOPPs) are also ligands for RAGE.20 AOOPs prepared in vitro (via incubation of native proteins with hypochlorous acid) or derived from in vivo sources (uremic serum) stimulated EC generation of superoxide, and activation of the following signaling mediators: NADPH oxidase, p44/42 and p38 MAP kinase, and NF-κB, all factors linked to increased expression of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1. Although pretreatment with anti-RAGE IgG or excess sRAGE mitigated these effects of AOOPs on cultured ECs, treatment with anti-AGE IgG had no effect, suggesting that AOOPs are unique non-AGE species. Other studies have suggested that oxidative stress produces AGEs.18,19

AGE-Counteracting Mechanisms:

Glyoxalase System

In addition to increased accumulation of posttranslationally modified protein species, disease states may be associated with decreased anti-AGE activities. Specifically, expression of glyoxalase I, an enzyme which detoxifies the pre-AGE methylglyoxal, is decreased in aging brain, Alzheimer’s disease brain, and aging lens.21,22 In the diabetic kidney, activity of glyoxalase is reduced, in part because of the decreased availability of a key cofactor, glutathione.23 Interestingly, although polymorphisms of the gene encoding glyoxalase I in human subjects demonstrated no relationship to vascular complications, one study reported relationships between glyoxalase I polymorphisms and prothrombotic events.24,25

In the context of RAGE, evidence suggests that RAGE regulates AGE formation and/or AGE detoxification, as RAGE-deficient mice display decreased levels of methylglyoxal and AGEs in such organs as the kidney and heart.26,27 The specific means by which RAGE protects from advancing AGE burden are under investigation.

In addition to posttranslationally modified species, certain members of the S100/calgranulin and HMGB1 families are signal transduction ligands of RAGE.1 How these ligands come face-to-face with RAGE appears to be a matter of cellular location, as both families appear to be largely located in the intracellular space in homeostasis.

S100/Calgranulins and HMGB1: Stepping Outside the Cell and Facing RAGE

In the search for putative “natural” ligands of RAGE, radio-labeled sRAGE was used to probe bovine lung extract, the material from which labeled AGE-BSA retrieved RAGE as a binding partner.1 The discovery that S100A12 and amphoterin (HMGB1) bound RAGE initially presented a quandary for several reasons: first, S100s and amphoterin were largely recognized as intracellular molecules.1 S100s were located in the intracellular space wherein at least in part through their “EF-hand” domains, these molecules mediated calcium signaling, phagocytosis and cellular migration.26 Amphoterin was described initially as a nonhistone DNA binding protein located in the nucleus.29 Second, the key signatures of S100s
and amphoterin struck the cords of inflammation, cellular migration and invasive potential. How might such molecules engage RAGE and, specifically, were such mechanisms related to diabetes?

In the case of S100s such as S100B, also a specific ligand of RAGE, S100B may exist in a soluble form in the intracellular space, and in association with intracellular membranes, centrosomes, microtubules, and type III intermediate filaments.\(^2\) In the intracellular space, the chief role of S100B is as a calcium sensor.\(^2\) Yet, much evidence indicates that S100B may be actively released from cells during various forms of stress, such as metabolic stress, physical exercise, and ischemia. Also, S100B may be “leaked” from damaged cells.\(^3\)\(^,\)\(^4\)

An analogous situation has been presented for the case of HMGB1. Although usually found in the nucleus in homeostasis, HMGB1 has been located at the leading edge of transformed cells and in neureite outgrowth.\(^3\)\(^,\)\(^5\) Furthermore, cells damaged by necrosis or autophagy, and those beset by poly(ADP)-ribose polymerase activation may release HMGB1.\(^3\)\(^,\)\(^5\)\(^,\)\(^6\) It is also possible that release of HMGB1 may be associated with cell death mechanisms initiated by apoptosis.\(^3\)\(^,\)\(^6\)

Irrespective of the mode of release from the intracellular space, experiments from multiple laboratories suggested that S100/calgranulin or HMGB1 interaction with RAGE-expressing cells modulated properties of inflammatory cells (monocytes/macrophages, T lymphocytes, and dendritic cells), vascular cells, epithelial cells, terminally differentiated cells such as neurons and cardiomycocytes, and transformed cells.\(^3\)\(^,\)\(^5\)\(^,\)\(^7\)\(^,\)\(^8\) These data supported the hypothesis that once “outside” the cell, S100/calgranulin and HMGB1 ligands may exert novel and profound effects on cellular phenotype. Via autocrine and/or paracrine mechanisms, such ligands may engage RAGE or other cellular receptors, thereby activating inflammatory and stress signaling pathways. Importantly, although suggested in vitro but not proved in vivo, the “dose” of these molecules may determine whether they mediate injury or contribute to repair. We predict that beyond the local concentrations available in the extracellular environment, the monomeric versus oligomeric forms of the ligands and their mode of presentation may be more important. For example, we recently demonstrated that multimeric forms of S100, particularly octameric forms, were most apt to stimulate RAGE in retinal pigment epithelial cells, activate NF-κB and increase expression of vascular endothelial growth factor.\(^3\)

Yet, given the balance of evidence, is it counterintuitive that evolution would perpetuate the expression and release of such molecules linked to injury? We propose that the timing, doses and forms of released S100/calgranulins and HMGB1 may be part of an exquisitely regulated system in which in acute stress, such ligand release may facilitate repair. However, once in the extracellular space, the vulnerability of these molecules to modification by oxidative, hyperglycemic and hypoxic stresses may result in their oligomerization, thereby increasing the likelihood that RAGE becomes their chief cell surface target.

In this context, certain S100/calgranulins and HMGB1 have been reported to bind to toll receptors as well as RAGE. Thus, it is conceivable that their occupancy of specific receptors may be mediated, at least in part, via the extracellular microenvironment into which they are cast consequent to cellular stress. The balance of evidence, to date, suggests that RAGE engagement amplifies inflammatory stress, down-regulates repair mechanisms and unless interrupted, causes cellular and tissue damage. Thus, it is not surprising that RAGE is expressed by multiple types of inflammatory cells, as these cells play central roles in the immediate responses to a diverse array of environmental stresses. In the sections to follow, we present the evidence linking RAGE to the immune response.

**RAGE and the Immune Response: Traversing a Fine Line Between Innate and Adaptive Responses**

The identification of S100/calgranulins and HMGB1 as RAGE ligands set the stage for addressing the hypothesis that RAGE played fundamental roles in inflammatory responses. RAGE is expressed in monocytes/macrophages and ligand-RAGE interaction contributes to central components of their biology, including migration, activation and delayed apoptosis in the face of ligand challenge.\(^5\)\(^,\)\(^4\) Recent evidence demonstrates that RAGE is expressed in T and B lymphocytes, and dendritic cells,\(^4\)\(^,\)\(^1\) thereby suggesting important roles for ligand-RAGE interaction in adaptive immune responses.

We first tested this hypothesis in a murine model of orthotopic allogeneic heart transplantation.\(^4\) Using allogeneic donors and recipients, we found that administration of sRAGE prolonged allograft survival in a dose-dependent manner compared to vehicle treatment. Histology of the grafts revealed a marked reduction in T lymphocyte and macrophage influx into the tissue in the presence of sRAGE.\(^4\) Although lymphocyte migration might have been one RAGE-dependent contributing factor, experiments in human and murine mixed lymphocyte reaction studies revealed that addition of anti-RAGE IgG or sRAGE, but not respective controls, attenuated proliferation of T lymphocytes.\(^4\) To more precisely address the specific RAGE-dependent mechanisms in T lymphocytes, OTII T lymphocytes (CD-4 like T lymphocytes expressing T cell receptors recognizing ovalbumin) were used. OTII mice were bred into the RAGE-null background. In both in vitro and in vivo studies RAGE-deficient OTII cells displayed significantly less proliferation and cytokine production (interferon-γ and interleukin-2) in response to ovalbumin versus RAGE-expressing OTII controls.\(^4\) We further probed roles for RAGE in dendritic cells. Contrary to distinct in vitro studies,\(^4\)\(^,\)\(^4\) we were unable to show specific roles for RAGE in vitro or in vivo in antigen presentation, maturation or migration properties.\(^4\) We concluded from these experiments that RAGE was required for effective T lymphocyte priming responses.

Homozygous RAGE-null mice and small molecule inhibitors of RAGE were then studied in an allograft murine model to further probe these concepts.\(^6\) Wild-type or RAGE-null mice were rendered type 1 diabetic with streptozotocin and then subjected to islet allografts. RAGE-null mice displayed delayed rejection compared to the wild-type counterparts and in wild-type mice, administration of small molecule RAGE
antagonists delayed rejection responses. Experiments in cell culture suggested that stimulated RAGE-null T cells displayed higher levels of interleukin-10 and interleukin-5, and that Interferon-γ production was reduced in wild-type stimulated T cells incubated with small molecule RAGE antagonists.46 Consistent with these data, higher levels of RAGE mRNA were found in clonal T cells subjected to Th1 differentiation conditions, thereby suggesting that RAGE activation in T cells may be a key factor in the early events linked to Th1 differentiation.46

The precise implications of these findings in specific biological and pathophysiological contexts are under active investigation. It is evident however that in infection or massive injury, at least in the murine system, deletion or antagonism of RAGE does not cause accelerated death or dramatic failure to clear pathogens such as mixed anaerobes in sepsis (cecal ligation and puncture), pneumococcal pneumonia, Listeria monocytogenes, and influenza A virus.47-50 In only one reported study to date, RAGE deficiency in the face of intraperitoneal injection of Escherichia coli was associated with reduced survival, suggesting that RAGE was required for effective endogenous immune responses against E coli.51 In chronic diseases, however, such as long-term diabetes, atherosclerosis or ventricular remodeling consequent to myocardial infarction, RAGE-impacted immune responses may actually be quite distinct. Perhaps in acute injuries, toll receptors or other innate immune compartments are activated whereas in chronic disease RAGE-dependent action in immune cells may sustain inflammation and exaggerate tissue damage. Indeed, reports suggest that RAGE ligands such as S100A8/A9 and HMGB1 may interact with toll receptors.52,53 Hence, the precise cellular- and microenvironment-specific context may directly regulate the biological fate of multi-receptor ligands. It is clear, however, that all of the ligands of RAGE identified to date share in common the ability to stimulate signal transduction. Recent studies have highlighted novel mechanisms in RAGE signaling.

**RAGE Signaling: Novel Intracellular Partners Facing the RAGE Cytoplasmic Domain**

Among the many cell types implicated in RAGE biology, a common feature is the ability of RAGE ligands to activate signal transduction cascades and induce cellular migration. The cytoplasmic domain of RAGE is essential for RAGE ligands to transduce their effects on gene expression and cellular properties. Truncation of the RAGE cytoplasmic domain in vitro or in vivo prevents RAGE ligands from activating signaling cascades and modulating expression and activity of central transcription factors involved in stress responses such as NF-κB and Egr-1.38 The specific means by which the RAGE cytoplasmic domain exerted these effects in the intracellular space were not known until recently, but appear to involve intermediate molecules in the cytoplasm. We probed a lung library in a yeast-two-hybrid assay system to identify binding partners of the RAGE cytoplasmic domain. A lung library was chosen as this tissue source was first used to isolate RAGE. In vitro- and in vivo-binding studies revealed that the RAGE cytoplasmic domain interacts with the formin homology 1 domain of mDia-1, a member of the formin family of molecules.54,55 As findings in yeast 2 hybrid assays may be nonspecific, it was essential to use multiple strategies to test the veracity of this interaction and to establish if mDia-1 was necessary for RAGE signaling. Hence, using tagged constructs and full-length RAGE expressing- versus RAGE cytoplasmic domain-deleted RAGE constructs in C6 glioma cells, we verified the interaction between the RAGE cytoplasmic tail and the formin homology 1 domain of mDia-1 and showed that the interaction was likely direct using an in vitro system.54 In our initial studies to probe the roles of mDia-1 in RAGE ligand-dependent signal transduction, we focused on major outcomes of RAGE signaling relevant to cellular migration and downstream activation of rac-1 and cdc42. First, we used C6 glioma cells, a model of transformed glial cells and showed that introduction of cytoplasmic domain-deleted RAGE suppressed RAGE ligand-mediated cellular migration and activation of rac-1 and cdc42. To directly address the ability of RAGE ligands to stimulate RAGE and downstream effector pathways, we used a second strategy in which RNAi was used to reduce mDia-1 expression in C6 glioma cells. Although downregulation of mDia-1 had no effect on RAGE expression, compared to scramble RNAi constructs, knockdown of mDia-1 significantly reduced RAGE ligand-stimulated cellular migration of C6 glioma cells. Importantly, knockdown of mDia-1 had no effect on a non-RAGE stimulus for cellular migration, such as fetal bovine serum. Furthermore, knockdown of mDia-1 impaired RAGE ligand-stimulated activation of cdc42 and rac-1, without affecting RAGE expression in the C6 glioma cells.54 These data set the stage for the testing of how RAGE ligands may signal through the cytoplasmic domain in vascular cells, and whether mDia-1 is required for RAGE signaling in these types of cells. Such studies are now in progress.

Of note, in another published study, it was reported that the RAGE cytoplasmic domain bound extracellular signal-regulated kinase (ERK). Although details were provided regarding the interaction of the 2 species, studies were not performed to test if loss of ERK or ERK function impacted the effects of RAGE ligands.56

Taken together, these findings delineate novel mechanisms by which RAGE stimulates signal transduction to induce changes in gene expression and phenotypes in cells under stress. It is possible, and likely, that the RAGE cytoplasmic domain binds to distinct intracellular molecules beyond ERK and mDia-1. Studies are in progress to delineate the full scope of mDia-1 roles in RAGE signaling, as specifically targeting the site of interaction between the RAGE cytoplasmic domain and the formin homology 1 domain of mDia-1 may underscore a new form of anti-RAGE therapies.

**Atherosclerosis and the RAGE Axis: Leading Roles for Multiple Ligands**

Atherosclerosis, particularly in diabetes, may be an important setting in which RAGE-regulated inflammatory responses contribute to acceleration of atherosclerotic plaque progression. In a detailed study of atherosclerosis in subjects who died suddenly, coronary arteries were retrieved and comparisons made between diabetic (type 2) versus nondiabetic age-
and ethnicity- and gender-matched controls. Several notable findings were made as follows.

First, the general observation was made that the type 2 diabetic subjects displayed larger mean necrotic cores and greater plaque load (both total and in the distal segments of the coronary arteries) than the nondiabetic subjects. Second, cellular composition was also different between diabetic and nondiabetic subjects with the diabetic lesions displaying more intimal staining for macrophages and T cells, as well as the activation marker HLA-DR. Third, the examination of RAGE and ligand S100A12 staining revealed higher expression of RAGE and S100A12 in diabetic versus nondiabetic lesions in association with more apoptotic macrophages and smooth muscle cells. Thus, increased inflammation (in parallel with increased ligand/RAGE expression) and larger necrotic core areas typified the diabetic lesions. Others found analogous findings in carotid artery atherosclerotic lesions. RAGE expression was higher in the diabetic versus nondiabetic lesions and the increased expression of RAGE was associated with increased oxidative and inflammatory stress compared to the nondiabetic lesions, including increased expression of matrix metalloproteinases. These findings closely paralleled experiments in cultured vascular and inflammatory cells in which ligand-RAGE interaction stimulated production of ROS, expression of inflammatory mediators, and cellular migration, all central features in the development and progression of atherosclerosis.

To date, in experimental models, 3 general RAGE-directed strategies have been used to test the role of RAGE in atherosclerosis. In the first strategy, mice vulnerable to the development of atherosclerosis were rendered diabetic either by administration of streptozotocin or by breeding apolipoprotein (apo)E-null mice into the db/db background, thereby generating models of type 1 or 2 diabetes, respectively. In both settings, induction of hyperglycemia had its greatest impact on vascular inflammation and oxidative stress. Mean atherosclerotic lesion areas were increased by diabetes both at the aortic sinus and throughout the aortic tree. Of note, in apoE-null or LDL receptor–null mice, induction of diabetes was also associated with a shift to proatherogenic lipid profile. Assessment of the role of RAGE was accomplished using the sRAGE decoy receptor. Administration of sRAGE resulted in dose-dependent reduction in accelerated atherosclerosis compared to vehicle treatment. Notably, sRAGE did not affect the levels of glucose or lipids in the animals, supporting the fundamental premise that the major effects of RAGE were on factors independent of traditional risk factors.

In the second strategy, homozygous RAGE-null mice were bred into the apoE-null or LDL receptor–null background and in both the nondiabetic or diabetic state, RAGE deletion caused a significant reduction in atherosclerosis and markers of inflammation in the vascular tissue. Levels of glucose and lipids did not differ between the RAGE-null versus wild-type mice irrespective of the diabetic state.

In the third strategy, transgenic mice specifically expressing cytoplasmic domain-deleted (dominant negative [DN]) RAGE in the endothelium were generated and bred into the apoE-null background and studied in the nondiabetic state. Transgene expression in the endothelium, but not in smooth muscle cells or macrophages, was verified before further study. Compared to apoE-null mice, transgenic endothelial DN RAGE mice displayed much less atherosclerosis. Ex vivo, functional monitoring of aortic rings from transgenic versus apoE-null mice revealed significantly better relaxation responses to acetylcholine, indicative of reduced endothelial dysfunction in the presence of the DN RAGE transgene in the endothelium.

To complement these studies, endothelial cells were retrieved from the aortas of wild-type, RAGE-null and endothelial DN RAGE mice. Incubation with RAGE ligands revealed that RAGE-null or endothelial DN RAGE mice endothelial cells were highly resistant to upregulation of inflammatory factors and matrix metalloproteinases. Interestingly, these experiments demonstrated that oxidized LDL contains AGE epitopes which at least in part transduce proinflammatory signaling via RAGE in endothelial cells. In the case of both S100B and oxLDL-AGE ligands, JNK MAP kinase signaling appears to importantly regulate RAGE-dependent inflammatory gene responses in murine aortic endothelial cells.

Finally, essential questions include the study of the effects of other RAGE-expressing cells in atherosclerosis, such as smooth muscle cells, monocytes/macrophages and T lymphocytes. Studies are underway to address these key questions as experiments in global RAGE-null mice may mask unique cell-type specific effects of RAGE in atherosclerosis.

Of note, the AGE hypothesis has been directly tested in cardiovascular disease as well. Administration of the AGE cross link breaker, ALT-711 (also known as alagebrum) to diabetic mice suppressed atherosclerosis and AGE accumulation in the vasculature. Other studies in animal models and human subjects support that use of this agent is effective in reducing the “AGEing” effects of diabetes and aging in reducing large artery stiffness, improving arterial compliance in elderly subjects, and improving cardiac function and uncontrolled systolic blood pressure in severely affected subjects. Thus, extensive evidence suggests that targeting AGE-RAGE in the vascular disease of aging and diabetes may be effective.

Hypoxia and Ischemia/Reperfusion: Rapid Generation of RAGE Ligands and Roles in Injury and Possibly Repair

In cardiac ischemia and reperfusion, studies have demonstrated that RAGE ligand expression and/or release is increased. Included among these are methylglyoxal pre-AGes, specific AGes such as carboxy methyl lysine AGE, S100B, and HMGB1. What is the impact of these ligands on the vulnerable heart? Distinct genetic and pharmacological strategies have been used to test this concept and the findings suggest that blunting the actions of RAGE is beneficial in the ischemia/reperfusion-stressed heart.

In the isolated perfused heart, irrespective of the state of hyperglycemia, administration of sRAGE to rats or mice before euthanasia resulted in cardioprotection, as evidenced by decreased release of lactate dehydrogenase, increased left ventricular developed pressure recovery and increased levels.
of RAGE-deficient mice hearts also displayed significant reduction in markers of cardiac injury compared to wild-type littermates, both in the presence and absence of diabetes. In the isolated perfused heart, consequent to ischemia and reperfusion, prominent expression of RAGE was noted in endothelial cells and in infiltrating monocytes/macrophages. To specifically test whether RAGE signaling in either of these cell types contributed to ischemia and reperfusion injury, we used transgenic mice expressing cytoplasmic domain-deleted RAGE in either endothelial cells or monocytes/macrophages. In both cases, when compared to wild-type littermates, the expression of the dominant negative transgene in endothelial cells or monocytes/macrophages significantly reduced ischemia/reperfusion injury.

In recent work, ischemia/reperfusion injury in the heart was induced in vivo via transient occlusion and reperfusion of the left anterior descending coronary artery. Administration of sRAGE or RAGE deletion resulted in significantly lower infarct volumes versus controls, in parallel with reduction in markers of apoptotic and necrotic injury. In the global left ventricular tissue, key roles for JNK and Jak/STAT signaling in RAGE-dependent ischemia/reperfusion injury were shown. Administration of RAGE ligand HMGB1 in this setting further increased cardiac injury, and injury did not occur when HMGB1 was administered to RAGE-null mice.

It is important to note that at least 4 studies to date have suggested that exogenous administration of HMGB1, or transgenic expression in the injured heart may exert benefit, perhaps by recruiting stem cells to assist in cardiac repair responses. Among the experimental conditions in all of these studies on HMGB1 and the heart in which the outcomes were divergent, there were significant differences in the type and duration of injury, animal species, dose of HMGB1, the precise mode of delivery or transgenic expression, and/or the specific injury, site and/or acuteness versus chronicity of perturbation dictate whether such ligands may be friend or foe.

Finally, it is important to discuss that fundamental roles for RAGE in ischemia/reperfusion injury are not limited to the heart. Rather, RAGE action is a common mediator of ischemia/reperfusion injury in other organs such as the brain, liver and lung. The detrimental consequences of RAGE may be mediated, in part, by its ability to upregulate Egr-1 in hypoxia. In the heart and liver, this mechanism may reflect a major common pathway in injury. Roles for RAGE in other oxygen-sensing and responsive pathway mechanisms are under active investigation at this time.

### Tracking the RAGE Axis in Human Subjects: Soluble RAGE and RAGE Polymorphisms

#### Soluble RAGE: A Biomarker of Disease Status and Response to Therapy?

Human subjects display soluble levels of RAGE detectable in plasma and body fluids. There are at least 2 forms of sRAGE, each generated by distinct mechanisms. First, Yonekura et al identified a novel soluble form of RAGE they termed "endogenous secretory” or endogenous secretory (es)RAGE. This material was detected in cultured endothelial cells and by virtue of an ELISA designed to capture the novel amino acids unique to this splice variant, esRAGE was found in human plasma. Later studies described the fuller range of human RAGE gene splice variants. Second, Bianchi and colleagues reported that a sheddase, ADAM10, cleaved membrane bound RAGE and others reported that matrix metalloproteinases could remove cell surface RAGE from the full-length receptor, thereby producing a circulating form of sRAGE, not expected to display the unique amino acids characterizing esRAGE.

Multiple publications have reported on findings associating levels of sRAGE with disease and/or disease status, including in the cardiovascular arena, particularly in diabetes. Furthermore, the effects of aging on sRAGE levels were addressed by Geroldi et al. This group studied a population of centenarians and reported that compared to healthy middle-aged persons and middle-aged persons with cardio-
vascular disease, levels of sRAGE were highest in the centenarians.90 Perhaps sRAGE is both a marker of healthy aging, and/or an innate protective mechanism. Much further work is required to address this key issue. Moreover, it is important to note that to date, very few published studies have examined both “total” sRAGE and esRAGE in the same patient populations, thereby missing potential opportunities to demonstrate that perhaps not total levels of sRAGES, but, rather, the relationship between total/esRAGE (ratios) might be predictive of disease vulnerability or status. Furthermore, it has been shown that increasing degrees of renal insufficiency lead to higher levels of sRAGE in affected persons, perhaps because of either more production and/or less efficient clearance.91

Interestingly, it has been reported that medications may alter levels of sRAGE, thereby reflecting caveats to be considered when levels of these species are reported in human subjects, even beyond renal function status.88,92 For example, treatment of type 1 diabetic subjects with perindopril increased sRAGE levels, and administration of telmisartan to subjects with essential hypertension resulted in decreased sRAGE levels.92 In another reported study, administration of atorvastatin over an 8-week period raised sRAGE levels in hypercholesterolemic subjects,92 and treatment of type 2 diabetic subjects with rosiglitazone raised total sRAGE and esRAGE levels over a 6-month period.92 So-called “nutraceuticals” such as grape seed proanthocyanidin extract have been reported to modulate sRAGE levels as well.92 It is important to note that to date no mechanisms have been proposed to explain why different medications (albeit in distinct subject populations) would have varying effects on sRAGE or esRAGE levels. Possibilities include effects of the medications on renal function, duration of therapy, or whether the therapeutic agents are true “modifiers” of disease or simply indirect treatments that target a consequence not a cause of major disorders. More research is clearly required to establish the mechanisms by which sRAGE and esRAGE are generated to best address these complex questions.

Figure. Glucose-igniting mechanisms leading to accelerated atherosclerosis and myocardial infarction: inciting RAGE in diabetes. Central to diabetes is the development of hyperglycemia. We predict that very early in glucose intolerance or diabetes, elevated levels of glucose surpass a threshold; once beyond that threshold, AGEs accumulate (I) and stimulate RAGE. One consequence of RAGE signaling is the activation of NAPDH oxidase (II) and production of ROS (III). Increased ROS may then contribute to increased production of AOPPs, more AGEs and AGE-modification of oxLDLs. Furthermore, increased ROS may deplete glutathione, thereby suppressing glyoxalase I activity, a mechanism favoring even further AGE accumulation (IV). AGEs, AOPPs, and AGE-oxLDL ligands of RAGE sustain stimulation of RAGE, and these processes, together with increased ROS, activate key transcription factors such as NF-κB and Egr-1 (V), which activate fundamental inflammatory mechanisms (VI). Consequences include increased migration and activation of RAGE-expressing neutrophils, monocytes/macrophages, T cells, and dendritic cells (VII). This results in release of the proinflammatory RAGE ligands S100/calgranulins and HMGB1. In this inflammatory environment, further AGEs may be formed as well. Via interaction with RAGE, these ligands magnify activation of NF-κB, Egr-1 and other factors (VIII), thereby amplifying cellular stress and tissue damage. In the aggregate, these processes may lead to accelerated atherosclerosis and increased myocardial damage in diabetes. Stopping the vicious cycle of RAGE ligand–RAGE interaction will be essential in curbing the maladaptive effects of glucose in diabetes. Illustration credit: Cosmocyte/Cameron Slayden.
Moving forward, large scale studies are needed to test the possibility that monitoring levels of total and esRAGE may establish novel biomarkers for tracking the diseases impacted by RAGE in the cardiovascular system and, fundamentally, in the inflammatory response.

RAGE Polymorphisms and Cardiovascular Disease

Our laboratory group extensively studied one of the coding change polymorphisms of RAGE, the G82S variant. This variant, within the major site of ligand binding, the V-type immunoglobulin domain, when expressed in cells, alters ligand (S100) affinity and increased ligand-stimulated generation of cytokines and matrix metalloproteinas.

However, the variant is relatively uncommon and even when we analyzed the Framingham offspring cohort, we were unable to identify any associations of this variant with cardiovascular disease.

Outside of the coding region, it is likely that variants in the promoter of RAGE may contribute to prediction for cardiovascular disease. For example, the −374T→A variant has been shown to be protective against the development of cardiovascular disease (T/A or A/A individuals) in both diabetic and nondiabetic individuals. Another variant in the promoter region, −429T→C, has also been studied. Unlike the −374T→A variant, however, no association has been reported with cardiovascular disease.

Importantly, however, many of the reported studies on RAGE polymorphisms in relation to cardiovascular disease are small. Furthermore, it is possible that haplotype analyses may be more informative in predicting the vulnerability to vascular disease, in the absence or presence of diabetes.

Taken together, evidence is mounting from both experimental model systems and human studies (tissue expression levels, sRAGEs and polymorphisms) to suggest that the RAGE axis is highly relevant to human disease.

Summary

Models of the pathogenesis of the complications of diabetes have evolved to explain how glucose exerts multiple maladaptive effects in vessels and tissues. In addition to high glucose-stimulated activation of the polyol pathway, protein kinase C and generation of reactive oxygen species via mitochondrial and NADPH oxidase-dependent mechanisms, the generation of AGEs ignites and stimulates multiple complications in the tissues. Cross-linking of long-standing proteins such as those in the basement membranes of the endothelium in the kidney and retina, for example, lead to vessel leakiness and loss of intercellular integrity. In addition, AGEs, via their interaction with their chief signaling receptor RAGE, spark activation of a host of maladaptive responses that fundamentally damage diabetic tissues.

Inextricably woven into the consequences of RAGE in the heart and great vessels is the finding that RAGE-dependent inflammatory mechanisms may modify the host response to superimposed injuries such as hyperglycemia, hypoxia, and ischemia/reperfusion. RAGE is expressed and active in neutrophils, monocytes/macrophages, T and B lymphocytes and dendritic cells: cell types that may affect immediate and late-stage responses to stress. To date, the balance of the evidence suggests that when RAGE is engaged, superinflammatory responses are set in motion which, if left undeterred, may irreversibly damage tissues. Hence, deletion of RAGE has been largely found to be protective, even in the face of pathogen onslaught or when massive doses of toxins and lipopolysaccharide are delivered to liver remnants after massive liver resection.

We predict that RAGE-stimulated inflammation aggravates diabetic tissues as well. Although of a chronic nature, these RAGE-dependent inflammatory perturbations nevertheless exaggerate inflammatory cell influx and activation into atherosclerotic plaques and the susceptible myocardium. Although the primary intent of these RAGE-dependent responses might be to rapidly respond to injury and perhaps, facilitate repair, they become entrapped in the vicious cycle of inflammatory injury and exacerbate it, not repair it. Coupled with failure or suppression of mechanisms to rid the diabetic tissues of inflammatory ligands, such as reduced glyoxalase 1, in part via RAGE, a chronic and vicious circle of injury is set in motion and sustained (Figure).

Clinical studies testing small molecule antagonists of RAGE are underway. Additional anti-RAGE strategies include sRAGE-like molecules as well as novel classes of antagonists to suppress interaction of the RAGE cytoplasmic domain with mDia-1. Tackling the RAGE axis in the diabetic heart and vessels is akin to diabetes itself: it is complicated! Only by identifying the specific settings in which RAGE antagonism might block maladaptive responses to glucosesparked stress and consequent inflammatory injury may we begin to test the hypothesis that the RAGE axis is a fundamental mechanism in the pathogenesis of diabetes and its complications.

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The RAGE Axis: A Fundamental Mechanism Signaling Danger to the Vulnerable Vasculature:
Correction

In the article that appears on page 842 of the March 19, 2010, issue, the figure legend should
contain a credit to the medical illustrator who prepared the image. The text should read as follows:

Illustration credit: Cosmocyte/Cameron Slayden.

This error has been noted in the online version of the article, which is available at
http://circres.ahajournals.org/cgi/content/full/106/5/842

Reference

1. Yan SF, Ramasamy R, Schmidt AM. The RAGE Axis: A fundamental mechanism signaling danger to the vulnerable

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