ANTIOXIDANTS & REDOX SIGNALING
Volume 7, Numbers 11 & 12, 2005

Forum Review

Diabetic Vascular Disease: It's All the RAGE

BARRY I. HUDSON, THORALF WENDT, LOREDANA G. BUCCIARELLI, LING LING RONG, YOSHIFUMI NAKA, SHI FANG YAN, and ANN MARIE SCHMIDT

ABSTRACT

The major consequence of long-term diabetes is the increased incidence of disease of the vasculature. Of the underlying mechanisms leading to disease, the accumulation of advanced glycation end products (AGEs), resulting from the associated hyperglycemia, is the most convincing. Interaction of AGEs with their receptor, RAGE, activates numerous signaling pathways leading to activation of proinflammatory and procoagulatory genes. Studies in rodent models of macro- and microvascular disease have demonstrated that blockade of RAGE can prevent development of disease. These observations highlight RAGE as a therapeutic target for treatment of diabetic vascular disease. *Antioxid. Redox Signal.* 7, 1588–1600.

INTRODUCTION

DIABETES MELLITUS is a chronic metabolic disorder characterized by an inherited and/or acquired deficiency in the production or effect of insulin. This deficiency results in an increase in blood glucose levels, which in turn may damage the vasculature and other target tissues in diabetes, such as the retina, kidney, and nervous system. Based on the last estimate by the World Health Organization (WHO), 150 million people are believed to have diabetes worldwide (89). By the year 2025, the WHO predicts that this figure will double due to increases in incidences of diabetes in developing countries and increases in obesity/sedentary lifestyles (88). The costs of treating diabetes and its associated complications have great implications, and therefore measures to prevent the development of both diabetes and its associated complications are needed. However, researchers are only now beginning to understand the molecular and cellular mechanisms underlying these pathologies, and how to translate basic biological findings to clinical therapies. One such mechanism is the increased accumulation of glycated proteins resulting from the increased glucose levels seen in diabetes, and upregulation of a receptor for these modified adducts, which drive the vasculature to a diseased state (8). This review will focus on the process of nonenzymatic glycation and the induction of vascular dysfunction via their central cell surface signal transduction receptor, RAGE [receptor for advanced glycation end products (AGEs)].

HYPERGLYCEMIA AND DIABETIC VASCULAR DISEASE: TOO MUCH OF A GOOD THING?

It has long been recognized that high glucose levels in diabetes is a major factor in the development of disease in the micro- and macrovasculature (50, 54). In 1993, the results of the Diabetes Control and Complications Trial confirmed these observations for the first time in a cohort of 1,441 type 1 diabetic subjects (76). In this study, it was revealed that strict control of glucose levels, as assessed by measurement of glycosylated hemoglobin, led to a 50–60% decrease in the incidence of microvascular disease (76). This obviously demonstrates the role for factors other than glycemia in diabetic vascular disease, especially in the setting of macrovascular disease. These same conclusions were made by the United Kingdom Prospective Diabetes Study. In that study, performed on 4,585 type 2 diabetic subjects, the relationship between glycemia and microvascular disease was confirmed,

Department of Surgery, College of Physicians and Surgeons, Columbia University, New York, NY.

and, in addition, a link between increased glucose levels and incidence of macrovascular disease was made (71). These studies demonstrated not only the association between hyperglycemia and vascular disease, but also that an increase in the nonenzymatic glycation of macromolecules may be a contributory mechanism driving disease pathogenesis.

A number of mechanisms have been demonstrated to play a role in the pathogenesis of diabetic vascular disease resulting from hyperglycemia (Fig. 1). These include increased flux through the sorbitol pathway via aldose reductase (79), increased activation of protein kinase C isoforms (90), and the formation of AGEs (7). All of these pathways seem to share a common link: the increase in stress-activated signaling pathways resulting in the increased production of reactive oxygen species leading to oxidative stress (8). Studies from Michael Brownlee's laboratory suggest these pathways are intertwined and result in the overproduction of superoxide by the mitochondrial electron transport chain, leading to oxidative stress and vascular dysfunction (8). The focus of this review is on the production of AGEs and their impact via the RAGE, a multiligand molecule implicated in a range of inflammatory disorders.

NONENZYMATIC GLYCATION AND VASCULAR DISEASE: A THEORY COMING OF AGE

So how does too much glucose, the most essential metabolite, result in irreversible vascular dysfunction from the nonenzymatic reaction of sugars with proteins? The idea of a nonenzymatic process, leading to spoiling, may not seem such a foreign concept to a food scientist. As early as 1912, the French chemist Louis Maillard noticed that when sugars were heated with amino acids, the building blocks of proteins, a nonenzymatic process occurred, resulting in a brown, pigmented solution (51). Food scientists demonstrated that this process could not only positively enhance the flavor of foods, such as gravy browning, but lead to the spoiling and aging of stored food (25, 51). This chemical pathway was not considered to be clinically relevant until analysis of hemoglobin subtypes revealed that a glycoyslated isoform (HbA_{1C}) was present in elevated levels in subjects with diabetes (78). Studies in rats demonstrated that under hyperglycemia, proteins of the lens became cross-linked and altered in color, a change similar to that seen with normal aging in the human lens, becoming more apparent in subjects with cataracts (15). It was then revealed that glycation of collagen occurred in the arterial wall leading to stiffening and vascular leakage, a hallmark of the initial phase of vascular disease, especially under conditions of hyperglycemia (10). The understanding of the chemistry of glycation from this prior work led to the development of the first compound to block the formation of AGEs (10).

The formation of AGEs is initiated by the nonenzymatic reaction between the carbonyl group of glucose and the amino group of a protein to form an unstable Schiff base, which undergoes rapid rearrangement to a more stable Amadori product (Fig. 2). These Amadori products are typically highly reactive dicarbonyls and over time, if not degraded, undergo rearrangement and oxidations to form a stable, irreversibly bound adduct known as an AGE.

The pharmacological inhibition of the glycation process was first performed using aminoguanidine (Pimagedine), a

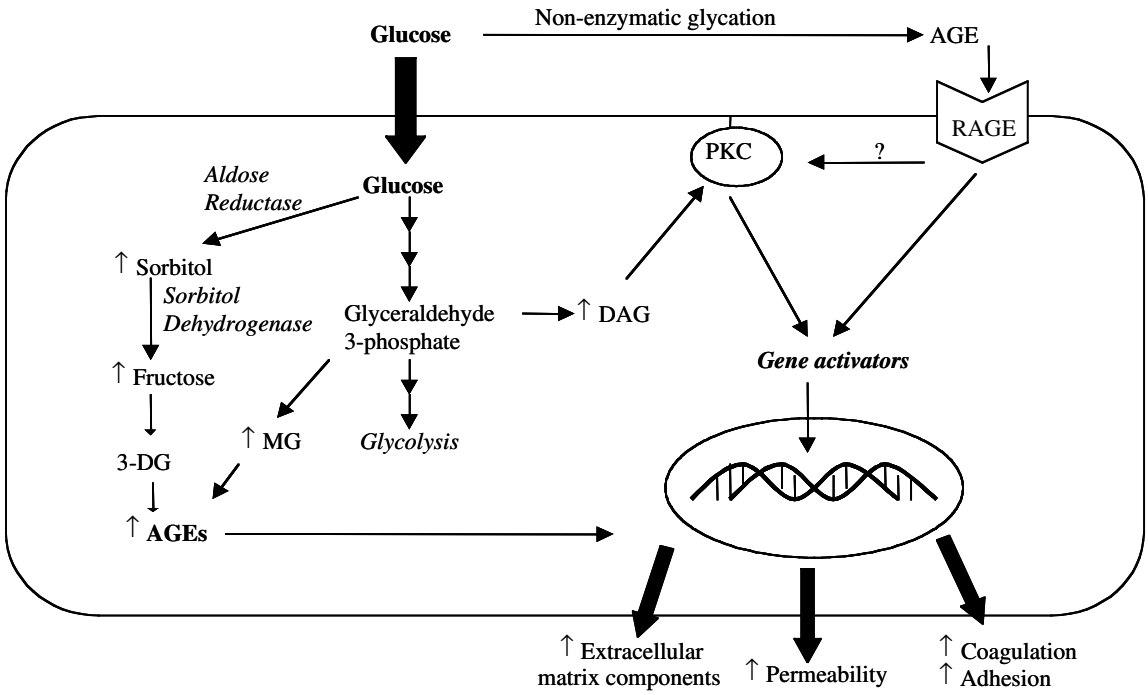

FIG. 1. Hyperglycemia-induced changes in the vasculature. Adapted from Feener and King (21). DAG, diacylglycerol; 3-DG, 3-deoxyglucosone; MG, methylglyoxal; PKC, protein kinase C.

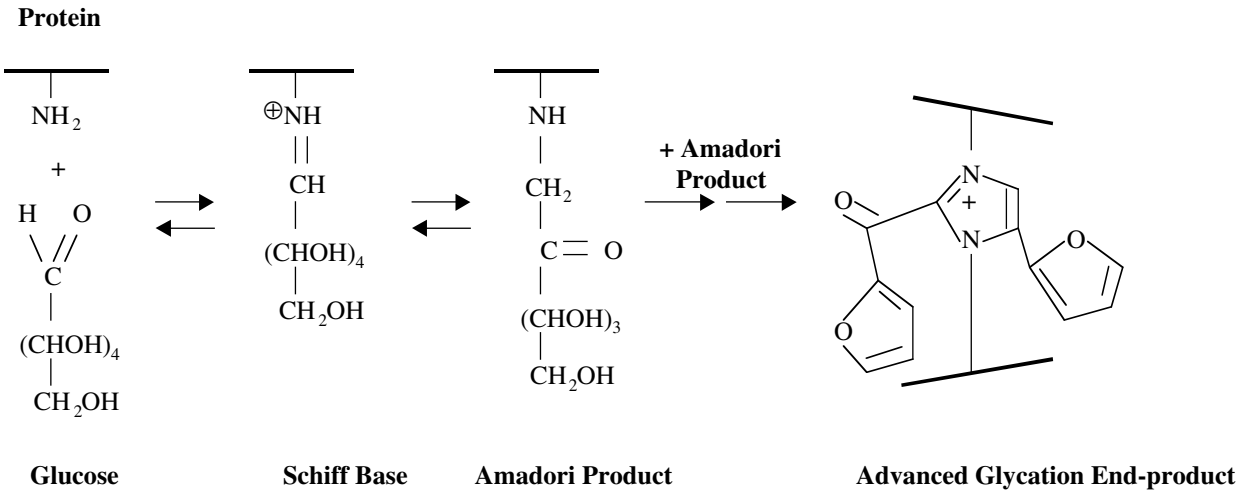

FIG. 2. The classical pathway of AGE formation. Glucose nonenzymatically reacts with an amine group of macromolecules (proteins, lipids, DNA) to form a reversible Schiff base. The Amadori product results from the unstable Schiff base rearranging into a more stabilized adduct. Additional reactions resulting from rearrangements, condensations, and dehydrations result in the formation of irreversibly bound AGEs. Adapted from Bierhaus *et al.* (5).

scavenger of the dicarbonyl, Amadori products (10). In an animal model of diabetes, aminoguanidine was able to block the arterial wall collagen cross-linking seen under hyperglycemic conditions (10). Further studies with this drug revealed that in experimental diabetes, development of microvascular complications, including neuropathy, nephropathy, and retinopathy, could be prevented (19, 23, 91). Other subsequent compounds have been developed that demonstrate similar properties, including pyridoxamine and ALT-711, a cross-link breaker of mature AGEs (14, 40). Initially, it was thought that AGEs mediated their effects through cross-linking of long-lived proteins, such as arterial collagen. However, recent research has extended the range of AGE structures formed, the metabolites involved, and the biological processes affected.

First, research has shown that not only are AGEs elevated in diabetic individuals, but also that they accumulate under conditions of oxidant stress, inflammation, and neurodegeneration (2, 68). Second, AGEs have been shown to form by multiple pathways and metabolites, including fructose, ascorbate, and various glycolytic intermediates more reactive than glucose (1, 87). This range of glycating molecules led to the generation of a heterogeneous population of different AGEs. However, only a number have been shown to be physiologically increased in their accumulation, which include *N*-(carboxymethyl)lysine (CML), pentosidine, and imidazolones (18, 66, 67). Third, the mechanisms that link the accumulation of these compounds to diabetic vascular disease have become clearer. Studies identified that not only could collagen cross-link other collagen molecules, but it could also lead to the trapping of extravascular proteins characteristically found in atherogenic plaques, thereby potentially contributing to the narrowing of the vascular lumen (9). AGE-collagen can react with and over time inactivate nitric oxide, a compound that regulates relaxation of smooth muscle and vascular tone (11). In addition, an enhancement in vascular permeability is seen with AGEs, and is preventable by treatment with aminoguanidine (80). Cross-linking of collagen has widespread implications, leading to alterations in the basement membrane, a common feature of vascular complications of diabetes. Changes in the expression of collagen in the glomerular matrix were demonstrated by the infusion of AGE into a nondiabetic rat model (95). These changes in gene expression were found to induce vascular dysfunction and glomerular lesions in these animals, reduced by the coadministration of aminoguanidine (95). A follow-up study (81) demonstrated that the long-term administration of AGE in the absence of hyperglycemia induced basement membrane thickening, mesangial extracellular matrix increase, and glomerulosclerosis, a typical feature of nephropathy (81). This was demonstrated to be AGE-specific by the amelioration of these effects by aminoguanidine. It was becoming evident from these studies that the effects of the AGE compounds were, to a large part, cell-mediated, implicating the role of an AGE-specific recognition factor on the cellular surface of the components of the vasculature, and hence the search began for an "AGE receptor."

RECEPTORS FOR AGES: A CELLULAR MECHANISM FOR REMOVAL AND/OR SIGNALING?

In 1989, Esposito and colleagues demonstrated the presence of AGE receptors on endothelial cells, and their ability to modulate endothelial cell permeability under prolonged AGE incubation (20). However, although these studies suggested a receptor-mediated mechanism of AGE induction, the specific cellular interaction site(s) were yet to be identified. Subsequent studies revealed that, not unlike the heterogeneous nature of AGEs, a variety of cell-surface receptors have been identified to bind AGEs, including the AGE receptor complex (OST-48, 80K-H, and galectin-3) (47, 82), macrophage scavenger receptors (MSR) type I and II (3), CD-36

(56), and LOX-1 (36). The majority of these receptors isolated to date have only demonstrated binding to highly glycated, *in vitro* produced AGEs. However, the first to be fully characterized, studied, and found to bind both *in vitro* produced AGEs and AGEs isolated from diabetic subjects is the receptor for AGE (RAGE) (55). Using bovine lung extract and AGE-albumin, RAGE was isolated and subsequently cloned (55). Homology analysis revealed RAGE to be a member of the immunoglobulin superfamily of receptors, due to its extracellular region containing one V-type immunoglobulin domain and two C-type immunoglobulin domains (55). The receptor was shown by hydropathy plot to contain a 332-amino acid extracellular domain, a single transmembrane spanning region, followed by a short, highly charged cytosolic tail (55). Truncation of this extracellular region demonstrated the ligand-binding domain of RAGE to reside in the V-domain (41, 64). A body of work now implicates RAGE as a signal transduction receptor after ligand engagement, as deletion of the cytosolic domain imparts a dominant-negative effect on ligand-induced gene activation (41, 63, 73).

One means by which AGEs elicit a toxic effect is by generation of reactive oxygen species. This occurs by a number of means, mainly via activation of RAGE (44). It was first seen that intracellular signaling from AGE/RAGE interaction leads to generation of reactive oxygen intermediates, the activation of p21ras, and a cascade of mitogen-activated protein kinases (MAPKs), resulting in nuclear translocation of nuclear factor-κB (NF-κB), a gene linked to the activation of multiple proinflammatory genes (44). The mechanism underlying the generation of reactive oxygen intermediates was found to be via activation of NADPH oxidase (85). Subsequent studies have identified ligand engagement of RAGE induces multiple signaling cascades in addition to MAPK, including cdc42/rac, a pathway involved in cell growth and migration (33), and stress-activated JAK/STAT (Janus kinase/signal transducer and activator of transcription) pathways (63) (Fig. 3). Although it has been reported that the cytosolic domain of RAGE directly engages extracellular signal-regulated kinase-1/2 (ERK1/2) MAPK (35), it is probable that this represents at best only one mechanism by which RAGE mediates intracellular signaling, as many pathways downstream of RAGE activation do not intersect with ERK1/2 MAPK targets.

RAGE is expressed in all tissues, including the kidney, heart, liver, and brain, the highest level being in the lung (6). At the cellular level, RAGE is found on the surface of most cell types, including endothelial, macrophage, neuronal, and glomerular epithelial cells (6). However, the striking feature of RAGE expression is that under normal homeostasis very low levels are detected, but under pathogenic scenarios such as diabetic vascular disease, a sustained up-regulation of RAGE levels is observed (6, 62, 75, 86).

MULTIPLE LIGANDS: THE MANY FACES OF RAGE

Another striking feature of RAGE biology is its ability to bind non-AGE ligands. This multiligand function is seen with

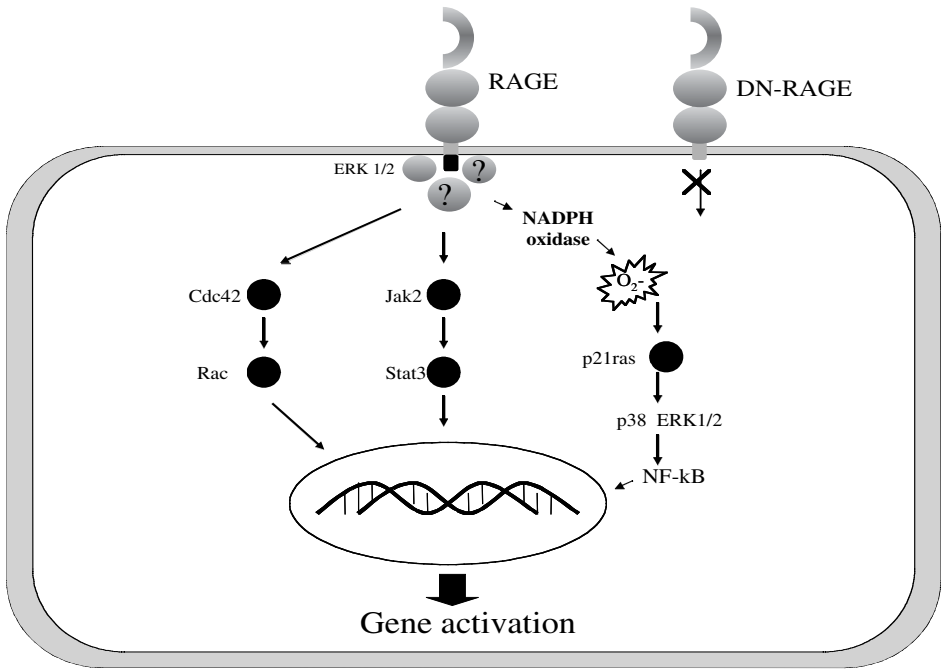

FIG. 3. RAGE-induced signaling pathways. The binding of ligands to RAGE (AGEs, S100/calgranulins, amphoterin) results in activation of a cascade of signaling pathways, including cdc42/rac, JAK/STAT, and other MAPKs, leading to gene activation and cellular effects. Expression of the dominant-negative (DN) form of RAGE, with the signaling tail removed, blocks signaling and gene expression.

other receptors of the immunoglobin family, including CD-36 (42), another AGE receptor. Subsequently, a number of non-AGE ligands have been shown to bind and function via RAGE, including the β-amyloid peptide, which accumulates in neuronal diseases such as Alzheimer's disease (94), amphoterin (28), S100/calgranulins (26), and most recently Mac-1 (16).

Amphoterin, more commonly known as high mobility group box 1 (HMGB1), was initially identified and studied as a nuclear protein (13). It is now well described as an extracellular mediator of cell migration and inflammation (22). Interaction with RAGE in developing neurons demonstrated a mechanism of migration and outgrowth (28). In tumor cells, this effect was exacerbated by the amphoterin/RAGE interaction, which enhanced cellular migration, invasion, and proliferation both *in vitro* and *in vivo*, and was blocked by the extracellular binding domain of RAGE (73).

The isolation of another RAGE ligand, termed extracellular newly identified RAGE binding protein (EN-RAGE), showed this molecule to be S100A12, a member of the S100/calgranulin family of proteins consisting of ~20 highly homologous proteins (26). Subsequent studies have demonstrated RAGE to function as a cellular mediator of many members of this family, including S100B (26), S100A1 (35), S100A4 (70), S100A8/9 (49), and most recently, S100P (4).

The recent identification of Mac-1 as a ligand for RAGE further expands the repertoire of RAGE biology (16). Chavakis *et al.* demonstrated that RAGE acts as a counter-receptor for the β2-integrin, Mac-1, mediating increased leukocyte adhesion in diabetic animal models (16). This represents a further means by which RAGE, which shares high sequence homology with other adhesion molecules, may enhance the recruitment of leukocytes in diseased tissues and amplify inflammatory responses.

RAGE AND VASCULAR DISEASE

In addition to the increased accumulation seen with AGE/RAGE in both animal models of diabetes and human diabetic subjects, immunohistochemistry showed that, in apolipoprotein E (apoE) null mice, atherosclerotic lesions were enriched not only in AGE/RAGE, but also in S100s (12). Research from Heiki Ravaula's group demonstrated that amphoterin and S100s signal through RAGE (33). Our group designed multiple studies to investigate the impact of RAGE/ ligand interaction on diabetic models of vascular disease, including its blockage and expression.

RAGE ENHANCES PERMEABILITY IN THE DIABETIC VASCULATURE

The first indicator of vascular disease in diabetic subjects is thought to be increased leakage of proteins in the vasculature (65). As a first test of the role of RAGE in vascular disease, we induced diabetes in rodents using streptozotocin (STZ), and after 11 weeks of hyperglycemia, these animals displayed increased vascular permeability (84). By measuring the tissue-blood isotope ratio, a threefold increase in vascular permeability was observed in the skin, kidney, and intestine in diabetic compared with nondiabetic animals (84). AGEs produced *in vitro* and isolated AGEs from human diabetic subjects were infused into both nondiabetic and diabetic rats (84). Blockade of ligand/RAGE interaction using polyclonal antibodies to RAGE and sRAGE (soluble RAGE, the extracellular domain of RAGE) blocked permeability in the vasculature. These studies demonstrated that blocking RAGE activation may prove to be a useful therapeutic tool to prevent diabetic vascular disease. We therefore pursued this further as to how RAGE impacted on a more severe manifestation of vascular disease.

RAGE IS A PROGRESSION FACTOR IN DISEASE OF THE MACROVASCULATURE

The major cause of mortality in diabetic subjects is macrovascular disease typified by atherosclerosis. To study this in animal models is difficult due to the high levels of cardioprotective high-density lipoproteins in rodents. To address this, we used apoE knockout mice made diabetic with STZ, which rapidly develop atherosclerosis. After 6 weeks of diabetes, these animals were examined for the presence of disease in the vasculature (58). Analysis of aortae revealed increased lesions that were more complex in the diabetic compared with the nondiabetic mice (58). In addition, staining for AGE/RAGE and S100s showed increased levels in diabetic aortae (12). At the onset of diabetes, mice were treated with sRAGE, which resulted in a dose-dependent suppression of accelerated atherosclerosis (58). In parallel, AGE accumulation was diminished. ApoE-null mice were bred into the db/db genetic model of diabetes to further confirm this result (12). These mice formed more aggressive arterial disease, which was blocked by treatment with sRAGE (12).

In addition, our data suggested that RAGE antagonism attenuated enhanced oxidative stress observed in the diabetic apoE null animals. When low-density lipoprotein was retrieved from the plasma of sRAGE-treated and vehicle-treated mice, we observed that susceptibility to copper-induced oxidation was decreased in sRAGE-treated animals (58). Based on increased lag time to copper-induced oxidation in sRAGE-treated low-density lipoprotein, we deduced that overall, diabetes-associated enhanced oxidative stress was attenuated by blockade of RAGE. These findings provided a direct *in vivo* correlate of previous studies carried out in cell culture and in nondiabetic animals infused with AGEs.

As atherosclerosis is a progressive disease spanning many decades, we tested whether blockade of RAGE could impact on established atherosclerosis. Using the apoE-null STZ diabetic mice, after 6 weeks of established diabetes, mice were treated with either sRAGE or placebo for a further 6 weeks (12). Analysis of aortae demonstrated that sRAGE stabilized lesion complexity and size, and reduced markers of vascular inflammation (12). Studies of the underlying cellular mechanisms revealed that sRAGE blocked both the proliferation

and migration of mononuclear phagocytes (MPs) and smooth muscle cells (SMCs) (12). To extend these findings to nondiabetic vascular disease, euglycemic apoE null mice were treated with sRAGE (12). In treated animals, sRAGE reduced atherosclerotic lesion area and complexity (12). In both the diabetic and nondiabetic apoE null animals, sRAGE reduced expression of RAGE, as well as levels of CML-AGE and S100s (12). These findings extend the concept of RAGE up-regulation in vascular disease in diabetes to have a role in the atherosclerotic process. Recent studies in human diabetic and nondiabetic subjects strongly support a role for RAGE in the atherogenic process (17). Atherosclerotic plaques retrieved from diabetic subjects revealed markedly higher levels of RAGE compared with those from nondiabetic subjects (17). RAGE levels also correlated with higher levels of glycosylated HbA_{1C} and an unstable plaque phenotype (17).

These data highlight the role for RAGE antagonists in the treatment of atherosclerotic disease, and thus we expanded our investigations to other processes involving atherosclerosis. In particular, we focused on the potential role of RAGE in restenosis, a complication arising after angioplasty. In diabetic subjects in particular, atherosclerosis occurs more rapidly and is more diffuse throughout the macrovasculature (63). Restenosis, an injury triggered by neointimal expansion, occurs more frequently in diabetic subjects. The major mechanism underlying restenosis is neointimal hyperplasia resulting from the proliferation of SMCs (83).

To study this and dissect the impact of RAGE-dependent mechanisms, we used diabetic animal models that demonstrated the accelerated expansion of the neointima and subsequent restenosis. Using hyperglycemic fatty Zucker rats, a balloon injury model to the carotid artery was employed (96). Administration of sRAGE to animals reduced the expansion of the neointima in concert with reduced RAGE expression (96). To test the role of RAGE in the nondiabetic state, we then performed femoral artery endothelial denudation injury in wild-type C57BL/6 mice (63). RAGE expression was increased in the femoral artery after 3 days of injury and was sustained until day 28 along with AGE and S100 levels (63). Injection of sRAGE to mice within the first 7 days of injury suppressed neointimal expansion in a dose-dependent manner (63). To confirm these results, we tested the impact of arterial injury in genetically altered RAGE mice (63). First, in homozygous RAGE-null mice, neointimal expansion was blocked (63). Second, using a transgenic model expressing the dominant negative-RAGE (DN-RAGE) form specifically in the SMCs, driven by the SM22α promoter, neointimal expansion was markedly reduced (63). These findings highlight that RAGE signaling contributes to smooth muscle proliferation in the expansion of the neointima (63). Using extracts taken from injured vessels, signaling pathways were examined to elucidate the underlying molecular mechanisms triggered by RAGE (63). This was found to involve activation of Jak2 and Stat3 leading to the up-regulation of tenacin-C and matrix metalloproteinase-12 (MMP-12), two genes involved in expansion and remodeling of the extracellular matrix (63). To extend these findings to an atherosclerotic state, we performed arterial injury in atherogenic prone apoE null mice. At the age of 12 weeks where hypercholesterolemia is present, sRAGE treatment blocked neointimal expansion (63).

RAGE AND COMPLICATIONS OF THE DIABETIC MICROVASCULATURE

Although macrovascular disease is the major cause of mortality of all diabetic complications, vascular disease can manifest itself in the microvasculature leading to blindness (retinopathy), kidney disease (nephropathy), and various peripheral neuropathies (53). Disease of the kidneys accounts for the highest rate of mortality of any microvascular complication (53). It has previously been shown that accumulation of AGEs in animal models of diabetes leads to the development of albuminuria (19). Studies in rats by Soulis *et al.* demonstrated that RAGE was up-regulated in the kidney with long duration of diabetes with accumulated AGE (69). Our own studies located the expression of RAGE to the glomerular epithelial cell, also known as the podocyte, in both human subjects with diabetes and animal models (75). Investigation of RAGE's role in podocyte activation and the subsequent increased excretion of albumin, leading to loss of renal function, was performed in the db/db type 2 diabetic mouse model (86). Histology confirmed in the db/db mouse that RAGE was present in the podocyte, with CML-AGE and S100 levels increased in MPs in the glomerulus (86). Mice were treated with sRAGE, which blocked expression of vascular endothelial growth factor (VEGF), a key mediator of hyperpermeability and MP recruitment/infiltration in the glomerulus (86). In homozygous RAGE null mice made diabetic with STZ, compared with wild-type mice, sRAGE also blocked VEGF expression, mesangial expansion and thickening of the glomerulus in early diabetes (86).

Previous studies by Yamamoto and colleagues demonstrated, in a double-transgenic mouse model of RAGE overexpression in the vascular endothelium and insulin knockout, that enhanced renal disease occurred compared with the single insulin knockout mice (93). Assessment of RAGE expression revealed significant expression of the RAGE transgene in monocytic cells (MPs) (93). Thus, it is possible that the impact of the RAGE transgene in augmenting nephropathy was due to expression in both the vascular endothelium and infiltrating MPs.

In addition to nephropathy, disease of the retina, although not a life-threatening condition, occurs in varying degrees in almost all diabetic subjects. RAGE expression was studied in specimens from patients with macular degeneration, along with CML-AGE. Histological examination revealed RAGE immunoreactivity in the diseased subretinal membranes, along with immunoreactivity for CML-AGE (24). Our studies in patients with proliferative retinal disease support these data; in addition, S100 and amphoterin were found to be up-regulated as well (57). Further studies are required to determine the cell/cell signaling mechanisms involved. Preliminary studies from the laboratories of Yamamoto's group show in microvascular endothelial cells that blocking RAGE/ligand interaction

prevents angiogenesis resulting from VEGF activation (92). Our own studies are currently focusing on novel *in vivo* animal models.

RAGE GENE VARIANTS: A PREDISPOSING FACTOR?

These data suggest that RAGE is an important gene involved in vascular disease pathogenesis, especially in diabetic individuals. Therefore, it is logical to study if allelic variants of RAGE may alter the expression and/or function of the molecules. In addition, data from epidemiological studies of diabetic subjects suggest a strong familial/genetic component in vascular disease pathogenesis (77).

The RAGE gene is found on chromosome 6 in the major histocompatibility gene complex, together with numerous inflammatory genes (72). Investigation of the protein coding region of RAGE revealed that within the 11 coding exons only one common variant exists: a Gly to Ser change at position 82 (29) (see Table 1 for all variants identified). This variant occurs in the ligand-binding domain of RAGE, and thus has subsequently been the focus of many studies (27, 29, 38, 43, 48). *In vitro* experiments to test the functional consequences of this variant revealed that the Ser82 isoform leads to increased binding and signaling via EN-RAGE (S100A12) and the increased activation of tumor necrosis factor-α, interleukin-6, and MMPs (27). Studies of the prevalence of the Ser82 allele in both macrovascular and microvascular disease have, however, not revealed any significant associations with disease propensity. Further studies are required in larger populations than those conducted so far due to the relatively low frequency of the Ser82 allele (5%).

The up-regulation of RAGE is a key event seen in vascular disease, and therefore gene variants in regions affecting RAGE expression itself could be important. Studies have identified that, within the −1,700 5′ flanking region of RAGE, a number of key positive and negative regions exist (45, 46, 74). Screening for polymorphisms within this region revealed numerous variants, most importantly two common single nucleotide polymorphisms proximal to the transcription start site (−374 T/A and −429 T/C) and a 63-bp deletion spanning from −407 to −345 (31) (Table 1). Importantly, part of the 5′ flanking region of RAGE overlaps with the PBX2 gene, which has a pseudogene copy on chromosome 3. This complicated the identification of the true polymorphisms within the RAGE gene, as highlighted by the −1,152 C/A variant (60), which was shown to be a gene:pseudogene difference (32).

Functional analysis using reporter gene assays revealed a two- (−429 C), three- (−374 A), and four- (63-bp deletion) fold increase over the wild-type sequence, with transcriptional binding assays demonstrating a loss of a transcription factor binding site to the −374 A allele (31). These compelling data suggested that these variants may affect the expression of RAGE levels, leading to increased disease pathogenesis. Studies to address this revealed no differences with respect to macrovascular disease (30), but a statistically higher prevalence of the −429 C allele (23.6% versus 14.9%) was seen in subjects with and without retinopathy, respectively (31). In a large population of 996 type 1 diabetic subjects with various degrees of nephropathy and poor metabolic control, the −374 A allele proved protective for both cardiovascular disease and lowered albumin extraction rate (59). Recent studies in South Indian subjects with type 2 diabetes demonstrate an association of the −374 A and −407 to −345 deletion allele with retinopathy (61). Together with the range of disease pathologies with which RAGE is associated, further studies are required to investigate the role of these functional variants on disease outcome.

CONCLUSION: RAGE AND BEYOND GLYCATION

In conclusion, our studies have revealed that a receptor we initially identified to bind glycated proteins is in fact a multiligand receptor with a likely role in numerous disease settings. Under conditions of hyperglycemia and subsequent oxidative stress, AGE formation is increased, leading to enhanced expression of RAGE and other RAGE ligands, to set the stage for long-term cellular activation and vascular dysfunction. Blocking this cascade of events by antagonism of RAGE not only slows the process, but also may reverse established disease in diabetic micro- and macrovasculature in small animal models. These considerations therefore highlight RAGE as a novel target for the treatment of the debilitating complications associated with long-term diabetes.

However, in order to fully predict the effects of long-term blockade of RAGE, it will be essential to delineate the homeostatic functions of RAGE. Initial observations in mice subjected to acute nerve crush injury suggested that blocking RAGE impairs nerve regeneration, thus highlighting innate roles for RAGE in both inflammatory and neurite-outgrowth promoting responses in the acutely perturbed peripheral nerve. Further studies are ongoing to unravel the homeostatic functions of RAGE in immunity and adaptation to cellular stress.

Certainly, dissection of the innate roles for this receptor in development and biology will be essential to uncover in order to fully elucidate the safety/efficacy of RAGE blockade in human subjects with chronic inflammatory diseases.

ACKNOWLEDGMENTS

These studies were funded, in part, by the Surgical Research Fund, United States Public Health Service, Juvenile Diabetes Research Foundation International, and Burroughs Wellcome Fund. B.I.H. is a recipient of a fellowship from the Juvenile Diabetes Research Foundation. A.M.S. is a recipient of a Burroughs Wellcome Fund Clinical Scientist Award in Translational Research.

ABBREVIATIONS

AGE, advanced glycation end product; apoE, apolipoprotein E; CML, *N*-(carboxymethyl)lysine; DN-RAGE, domi-

TABLE 1. ALLELIC VARIANTS OF THE RAGE GENE

RAGE allelic variant	*Region of gene detected*	*Allele frequencies*	*Disease association studies*	*Functional implications*	*References*
−1,420 (GTT)n	5′ flanking	N/A			31
−1,393 G/T	5′ flanking	N/A			31
−1,390 G/T	5′ flanking	N/A			31
−1,202 G/A	5′ flanking	N/A			31
−405 to −345 deletion	5′ flanking	>99% Ins, <1% Del		Increased expression in reporter gene studies.	31
−429 T/C	5′ flanking	83% −429T, 17% −429 C	No associations with ischemic heart disease in both diabetic and nondiabetic individuals. Association with retinopathy in diabetic individuals (24% −429C, $p = 0.012$).	Increased expression in reporter gene studies.	30–32, 59
−374 T/A	5′ flanking	81% −374T, 19% −374 A	No associations with ischemic heart disease in both diabetic and nondiabetic individuals. No association with retinopathy in diabetic individuals. Increased frequency in non–small cell lung cancer (39% −374A, $p < 0.05$) and subjects with proteinuria.	Increased expression in reporter gene studies. Altered binding of nuclear proteins.	30–32, 59
Ala2Ala (GCT/GCA)	Exon 1	86% T, 14% A			29
67 C/G	Intron 1	83% C, 17% G			29
Lys37Ser	Exon 2	>99% Lys37, < 1% Ser37			29
Arg77Cys	Exon 3	>99% Arg77, < 1% Cys77			29
Gly82Ser	Exon 3	95% Gly82, 5% Ser82	No association with macrovascular disease in diabetic and nondiabetic subjects. No association with diabetic microvascular disease. Increased frequency of Ser82 in diabetic skin disorders.	Increased ligand affinity and cytokine activation with Ser82 allele in macrophages.	29, 38, 39, 43, 48, 60
Val89Val (GTG/GTC)	Exon 3	95% G, 5% C			29
Gly90Gly (GCT/GCA)	Exon 3	95% T, 5% A			29
718 G/T	Intron 3	92% 718 G, 8% 718 T			39
Thr187Pro	Exon 6	>99% Thr187, <1% Pro187			39
1,704 G/T	Intron 7	95% 1,704 G, 5% 1,704 T	No association with diabetic retinopathy. Association with antioxidant status in type 2 diabetic subjects.		37, 39

Continued

TABLE 1. *CONTINUED*

RAGE allelic variant	*Region of gene detected*	*Allele frequencies*	*Disease association studies*	*Functional implications*	*References*
A insertion 1,727	Intron 7	N/A			39
His305Gln	Exon 8	>99% His305, <1% Gln305			29
Ser307Cys	Exon 8	>99% Ser307, 1% Cys307			39
Gly329Arg	Exon 8	>99%Gly329, <1%Arg329			39
2,117 A/G	Intron 8	N/A			39
2,184 A/G	Intron 8	84% 2184 A, 16% 2184 G	No association with diabetic retinopathy. Association with antioxidant status in type 2 diabetic subjects. Association of the 2,184G allele and plaque psoriasis and diabetic nephropathy.		37, 39
2,224 A/G	Intron 8	N/A			39
2,245 G/A	Intron 8	92% 2,245 G, 8% 2245 T	No association with diabetic retinopathy.		39
2,249 A/G	Intron 8	N/A			39
2,741 G/A	Intron 9	N/A			39
Leu363Leu (CTG/TTG)	Exon 10	99% C, 1% T			39
Arg389Gln	Exon 10	>99% Arg389, <1% Gln389			39
CA deletion 3089	3′UTR	<1% CA deleted			39

Important variants are highlighted in bold. Del, deleted; Ins, inserted; 3′UTR, untranslated region.

nant negative-RAGE; EN-RAGE, extracellular newly identified RAGE binding protein; ERK, extracellular signal-regulated kinase; JAK/STAT, Janus kinase/signal transducer and activator of transcription; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; MP, mononuclear phagocyte; NF-κB, nuclear factor-κB; RAGE, receptor for advanced glycation end products; SMC, smooth muscle cell; sRAGE, soluble RAGE; STZ, streptozotocin; VEGF, vascular endothelial growth factor; WHO, World Health Organization.

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Address reprint requests to:
Barry I. Hudson, Ph.D.
College of Physicians and Surgeons
Columbia University
630 W 168th Street
P&S 17-401
New York, NY 10032

E-mail: bh2021@columbia.edu

Received after revision May 27, 2005; accepted June 27, 2005.

This article has been cited by:

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