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THE RECEPTOR FOR ADVANCED GLYCATION END-PRODUCTS AND ATHEROSCLEROSIS: FROM BASIC MECHANISMS TO CLINICAL IMPLICATIONS

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Abstract

The receptor for advanced glycation end-products (RAGE) is a member of the immunoglobulin superfamily of cell surface molecules with a diverse repertoire of ligands. In the atherosclerotic milieu, three classes of RAGE ligands, i.e., products of nonenzymatic glycoxidation, S100 proteins and amphoterin, appear to drive receptor-mediated cellular activation and, potentially, acceleration of vascular disease.

The interaction of RAGE-ligands effectively modulate several steps of atherogenesis, triggering an inflammatory-proliferative process and, furthermore, critically contributing to propagation of vascular perturbation, mainly in diabetes. RAGE has a circulating truncated variant isoform, soluble RAGE (sRAGE), corresponding to its extracellular domain only. By competing with cell surface RAGE for ligand binding, sRAGE may contribute to the removal/neutralization of circulating ligands thus functioning as a decoy. The critical role of RAGE in the chronic vascular inflammation processes highlights this receptor-ligand axis as a possible and attractive candidate for therapeutic intervention to limit vascular damage and its associated clinical disorders.

Key Words

Receptor for advanced glycation endproducts; atherosclerosis; S100 proteins; amphoterin.

Introduction

The receptor for advanced glycation end products (RAGE) is a multiligand member of the immunoglobulin superfamily of cell surface molecules, that was first described as receptor for adducts modified by non-enzymatic glycosylation occurring on proteins and lipids in a wide variety of setting, mainly in diabetes[1, 2]. The ability of RAGE to recognize a wide-range of endogenous ligands, such as advanced glycation end products (AGEs), S100 proteins, amphoterin and others[3-8], suggests that this receptor may function as an excellent sensor for the environmental signals and hence play a crucial role in the regulation of homeostasis and pathogenesis. This review focus on the pathogenetic role of RAGE-ligand interaction and its implication in the development and progression of atherosclerosis, principally in diabetes.

Chronic inflammation provides the basis for many complex diseases including atherosclerosis[9, 10]. The expression of adhesion molecules on endothelial cell surface is an early, and necessary, step in the pathogenesis of atherosclerosis[11]. In response to signals generated within the early lesion, monocytes adhere via adhesion molecules to the endothelium and then migrate into the intima by producing enzymes, including locally activated matrix metalloproteinases (MMPs) that degrade the connective tissue matrix. The activation of monocyte leads to both local release of monocyte-colony stimulating factor, which causes monocytic proliferation, and cytokine-mediated progression of atherosclerosis. Attraction of inflammatory cells such as monocytes, polymorphonuclear leukocytes, and T-lymphocytes into the vessel wall offers a mechanism to protract the inflammatory response. Smooth muscle cells (SMC) are other important actors in the pathogenesis of atherosclerosis. During plaque formation SMC migrate from the media toward the intima, where they proliferate and undergo phenotypic changes. The impact of the mediators

released by inflammatory cells and SMC is various and includes mitogenesis, angiogenesis and foam cell development.

In the context of RAGE, that is expressed in all cell types relevant to the development of atherosclerotic plaque (i.e., endothelial cells, SMC, monocytes/macrophages, and lymphocytes)[12, 13], activated inflammatory cells may release mediators/RAGE-ligands such as S100 proteins and amphoterin. Once these molecules are released into the vessel wall, S100 proteins/amphoterin–RAGE interaction may amplify tissue inflammation and injury by autocrine and paracrine pathways[6].

Enhanced RAGE expression in human diabetic atherosclerotic plaques colocalize with cyclooxygenase-2 (COX-2), type 1/type 2 microsomal Prostaglandin E2, and MMPs, particularly in macrophages at the vulnerable regions of the atherosclerotic plaques[13]. The overlapping accumulation and expression of RAGE and its ligands at sites of tissue lesions sustains a RAGE-mediated cellular activation and propagation of inflammation in this established disease. Although the RAGE-ligand axis provide an important role to accelerated atherosclerosis in diabetes, several recent evidences highlight that it may contribute to chronic and amplified inflammatory status in atherosclerotic processes also beyond diabetes.

RAGE structure, polymorphisms and vascular expression

RAGE variants. RAGE is highly conserved across species and expressed in a wide variety of tissues: it is most abundant in the heart, lung, and skeletal muscle[2]. In the vessel wall, the RAGE is localized in the endothelium, SMC, monocytes[14, 15]. RAGE is an approximately 45-KDa protein originally isolated from bovine lung endothelium on the basis of its ability to bind AGE ligands[1]. Subsequent molecular cloning revealed RAGE as a newly identified member of

the immunoglobulin superfamily of cell-surface molecules[2]. The entire mature receptor consists of 403 aminoacids in man, rat and mouse. The extracellular region of RAGE consists of one V-type (variable) immunoglobulin domain, followed by two C-type (constant) immunoglobulin domains stabilized by internal disulfide bridges between cysteine residues. The V-type domain includes two putative N-linked glycation sites. It has been recently shown that the V and C1 domains are not independent domains, but rather form an integrated structural unit. In contrast, C2 is attached to VC1 by a flexible linker and is fully independent [16]. In addition to the extracellular domain, RAGE also contains a short hydrophobic domain that corresponds to a single transmembrane domain, which is followed by a short cytoplasmic domain that is essential for RAGE-mediated signal transduction[2, 17]. In addition to full-length RAGE, several truncated forms of the RAGE receptor have been described[18-22] (**Fig. 1**). In particular, two major RAGE mRNA splice variants have been thoroughly characterized. One variant protein (N-truncated type) lacks the V-type immunoglobulin domain, but it is otherwise identical to full-length RAGE and is retained in the plasma membrane[19]. As a result of the deletion of the V-type immunoglobulin domain, this variant protein is significantly impaired in its ability to bind RAGE ligands[19]. The other variant (C-truncated type), containing the same immunoglobulin domains present in full-length RAGE, but lacking the cytosolic and transmembrane domains[19], is secreted extra-cellularly and can be detected in circulating blood. To add to this complexity, three novel human RAGE transcripts all encoding truncated soluble form of RAGE (sRAGE), have been identified[20]. In the last section of this review the role of the secretory form of RAGE is examined in detail.

RAGE polymorphisms. The RAGE gene is located on chromosome 6 in the major histocompatibility complex locus in the class III region[23]. The RAGE gene is composed of 11

exons and a 3'UTR region [23], and among these exons there are a common variant in exon 3 (Gly82Ser) and 3 rare coding changes (Thr187Pro, Gly329Arg, Arg389Gln)[24]. As the Gly82Ser polymorphism is located in the ligand-binding V-domain of RAGE [24], it was studied to assess its function and prevalence in subjects with vascular disease. Cells bearing the Ser82 isoform displayed not only higher ligand affinity for S100A12 protein but also led to the increased activation of the proinflammatory proteins TNF-α, IL-6, and MMP-9, under stimulation with S100A12 [25]. These studies, therefore, suggest that the RAGE Gly82Ser polymorphism may influence inflammatory processes and hence play a role in the development of vascular disease.

Another interesting variant is the functional −374T/A polymorphism located in the promoter region of the RAGE gene that has shown to exert significant effects on transcriptional activity[26]. In type 1 diabetic patients with AA genotype, there was a lower incidence of coronary heart disease, acute myocardial infarction and peripheral vascular disease [27]. Similarly, in non-diabetic individuals, the AA genotype showed to be independently associated with a reduced risk of coronary artery disease[28].

RAGE expression. There is also significant evidence that the RAGE signalling pathway can be initiated by a diverse repertoire of ligands, including AGEs, S100 proteins, amphoterin, amyloid-beta peptide, β-sheet fibrils and, most recently, the leukocyte β_2-integrin MAC-1[3-8]. Apart from being a multiligand receptor, another unusual feature of RAGE is the co-expression with its ligands in tissues. Contrary to other receptors, such as the low density lipoprotein (LDL) receptor, which are downregulated by increased levels of their ligands, the RAGE-ligand interaction would thus lead to a positive feedback activation, which further increases receptor expression[3]. Beyond its ligands, other cytokines and proinflammatory molecules can upregulate the RAGE expression. TNF-α seems to be involved in the enhancement of RAGE expression in neointimal

formation[29], and C-reactive protein at concentrations known to predict future vascular events, up-regulates RAGE expression in human endothelial cells at both protein and mRNA level[30]. Instead, the peroxisome proliferator-activated receptor gamma (PPARγ) agonist rosiglitazone, that inhibits neointimal hyperplasia, has been shown to significantly down-regulate RAGE expression and to inhibit SMC proliferation in response to the RAGE agonist S100 proteins[31]. In vivo studies showed that rosiglitazone decreased RAGE expression and SMC proliferation at seven days following carotid arterial injury in both diabetic and nondiabetic rats, suggesting that down-regulation of RAGE by the PPARγ activation inhibits neointimal formation in response to the arterial injury[31].

The vascular ligands of RAGE

The RAGE is a receptor that may be activated by several proinflammatory ligands, of which those implicated in the atherosclerotic process are AGEs, S100 proteins and amphoterin[3, 6, 32]. AGEs are complex, heterogenous molecules generated by glycation and oxidation in vivo. Protein glycation (also known as the Maillard reaction) occurs between reducing sugars and free amino-groups of a protein via nucleophilic addition that forms a Schiff base. The labile Schiff base rearranges to form a stable, and essentially irreversible Amadori product. During Amadori reorganization, reactive intermediate carbonyl groups accumulate. These compounds are known as α-dicarbonyls or oxoaldehydes and include such products as 3-deoxyglucosone and methylglyoxal[33]. The α-dicarbonyls have the ability to react with amino, sulfhydryl, and guanidine functional groups in proteins[34], resulting in denaturation, browning, and cross-linking of the targeted proteins[34, 35]. In addition, the α-dicarbonyls can react with lysine and

arginine functional groups on proteins, leading to the formation of stable AGE compounds, such as N-ε-(carboxymethyl)lysine-adducts (CML). The identified AGEs can be classified into three major groups: fluorescent cross-linking species (e.g., pentosidine and crossline), non-fluorescent cross-linking species (e.g., arginine-lysine imidazole), and non-fluorescent, non-cross-linking species (e.g., pyrraline and CML). All three AGE classes have been identified and isolated from tissue, serum, and urine samples [36]. AGEs accumulate continuously on long-lived vessel wall proteins, and are present in the intima, media, and adventitia[37], prevalently in diabetes[38, 39]. When AGEs formation is enhanced, such as in animal models of diabetes[40] and patients with diabetes[15, 36], they are particularly abundant at sites of atherosclerotic lesions. This may be related to the pro-oxidant environment present in lipid-rich vascular lesions because AGEs formation is accelerated under conditions of increased local oxidant stress[12, 41, 42].

RAGE was initially identified as a receptor for CML adducts, which have been identified as the major non-fluorescent AGEs in vivo[43, 44]. CML also form in vitro from LDL incubated with copper ions and glucose and therefore are believed to be both lipid and protein adducts[45]. CML can also be generated on proteins by a myeloperoxidase-dependent pathway when neutrophils are activated[46]. This pathways of CML formation suggest that a direct link of inflammation to AGE formation exists. Until now, among the wide variety of AGE structures, only CML adducts have been identified as signal-transducing ligands for RAGE, both in vitro and in vivo[47].

Later, it was found that RAGE also interacts with other non-glycated endogenous peptide ligands such as S100 proteins[6] and amphoterin[3] that structurally have very little in common.

S100 proteins are calcium-binding proteins found in any inflammatory lesion, including the blood vessel wall of individuals with diabetes[48-50]. These proteins undergo a conformational change upon calcium binding, allowing them to interact with target molecules and initiate

biological response. In the specific context of inflammation and RAGE, these species may be produced in granulocytes, dendritic cells, monocytes, and lymphocytes[51-53]. At least seven members of the S100 family, S100A12, S100A1, S100A4, S100A11, S100A13, S100B, and S100P have been identified to be ligands of RAGE[6, 53, 54]. Among these molecules, S100A12 (also named calgranulin C or extracellular newly-identified RAGE binding protein) is predominantly expressed and secreted by early recruited neutrophils, and it seem to play a pivotal role in innate immune responses. Recently it has been shown that unlike AGEs, which bind to the V-type domain of RAGE[6], Ca2+ binding creates two symmetric hydrophobic surfaces on S100A12 protein that allow S100A12 to bind to the C-type immunoglobin domain of RAGE[55]. S100A12 binds RAGE in a saturable and dose-dependent manner on cultured endothelial cells, SMC, monocytes/macrophages, and lymphocytes[6] and it has been identified as signal transduction ligand for RAGE. In cultured human THP-1 macrophages, the level of mRNA/protein of S100A12 was induced by IL-6 and inhibited by the activation of PPARγ[56]. Probably the most relevant pro-inflammatory effects of S100A12 protein are related to the interaction of phagocytes with endothelium[57]. The excessive release at sites of inflammation observed in vivo is feasible to induce a positive feedback loop in which, primed phagocytes and endothelium stimulated by S100A12, facilitate the further recruitment of even more phagocytes.

The amphoterin, another proinflammatory ligand of RAGE, is a nuclear protein constitutively expressed in quiescent cells[58], and released passively from necrotic or damaged cells[59, 60]. Following its release the amphoterin is capable of inducing an inflammatory response, thereby transmitting the ‘injury’ signal to neighboring immune cells[59]. In addition to its passive release, it has been recently discovered that amphoterin may also be released from activated macrophages, in response to exogenous and endogenous inflammatory stimuli[61-63]. Therefore,

amphoterin might be a critical molecule that allows innate immune cells to respond to both infection and injury, thereby triggering a rigorous inflammatory response. In the pathogenesis of atherosclerosis the amphoterin, which is abundantly expressed in vascular endothelial cells, can be passively released from injured endothelial cells. Once released, extracellular amphoterin can stimulate neighboring endothelial cells to express various proinflammatory cytokines, chemokines (e.g. IL-8, monocyte chemotactic protein-1[MCP-1]), adhesion molecules (e.g. intercellular adhesion molecule-1 [ICAM-1] and vascular cell adhesion molecule-1[VCAM-1]), as well as RAGE[64-67] (**Fig. 2**). These adhesion molecules enforce strong attachment of infiltrated macrophages/monocytes to the endothelium, which should in turn further increase the release of amphoterin and other cytokines. In agreement with the above hypothesis, a dramatic increase in amphoterin levels has been observed in atherosclerotic lesions[68].

AGEs, S100 proteins, and amphoterin may activate cell types intimately involved in macro/microvascular disease initiation/progression, and given the diversity of proinflammatory ligands that RAGE could bond, it results evident that the pathophysiological role of this receptor can extend widely beyond diabetes.

Cellular effects of RAGE-ligand interaction

The most important pathological consequence of RAGE engagement with its ligands appears to be cellular activation, leading to the induction of oxidative stress and a broad spectrum of signalling mechanisms. The RAGE-ligand interactions lead to prolonged inflammation, mainly as a result of RAGE-dependent expression of proinflammatory cytokines and chemokines. In the vasculature, the first pathological consequence of RAGE interaction with its ligands is the

induction of increased intracellular reactive oxygen species (ROS)[69, 70], the generation of which seems to be linked, at least in part, to the activation of the NAD(P)H-oxidase system[71]. I and my colleagues have shown that, in endothelial cells, mitochondrial sources of ROS are also evoked secondary to AGE-RAGE interaction[72].

Experimental evidences demonstrate that RAGE-dependent modulation of gene expression and cellular properties depends upon signal transduction. Depending on the intensity/chronicity of ligand stimulation, diverse signalling pathways may be triggered. Signalling cascades activated upon ligand–RAGE interaction include pathways such as p21ras, erk1/2 (p44/p42) mitogen-activated protein kinases (MAPKs), p38 and SAPK/JNK MAPKs, rho GTPases, PI3K, and the JAK/STAT pathway; downstream consequences such as the activation of the key transcription factors, nuclear factor-κB (NF-κB) and cAMP response element binding protein have also been reported[6, 47, 73-76]. Each of these pathways link closely with ligand binding to RAGE, because the blockade of the receptor with either anti-RAGE IgG (which blocks the access of ligands to RAGE) or excess of sRAGE (the extracellular receptor domain which, competing with cell-surface RAGE for ligand binding, functions as a decoy) prevents their activation. Importantly, the tethering of ligands to the cell-surface is not enough to induce cellular activation, since the RAGE carboxy-terminal cytosolic tail, involving known signalling phosphorylation sites, and kinase domains, is critical for RAGE-dependent cellular activation. A RAGE mutant, lacking only the cytosolic tail and expressed in cells, retains binding to various ligands identical to wild-type RAGE, but does not mediate the induction of cellular activation[6]. Even if AGEs were nothing more than accidental ligands for RAGE, interaction of RAGE with the other ligands such as amphoterin or S100A12 protein induces similar consequences. The presence of RAGE in all cells relevant to atherosclerosis, including endothelial cells, monocyte-

derived macrophages, lymphocytes, and SMC, suggests the relevance of RAGE engagement in these processes (**Fig. 3**).

In human endothelial cells, AGEs or S100A12 induce the expression of adhesion molecules, including VCAM-1, ICAM-1 and E-selectin[77-79], in a RAGE-dependent manner, as confirmed by the inhibitory effect of anti-RAGE IgG or sRAGE. AGE-bound RAGE on the endothelium also determine alterations of the surface antithrombotic properties to the flowing blood, as shown by a reduction of thrombomodulin expression and the concomitant induction of tissue factor expression[80, 81]. Tissue factor induction and the reduced thrombomodulin activity change the dynamic endothelial properties from those of an anticoagulant to those of a procoagulant surface.

The interaction of AGEs with monocytes induces a phenotype of activated macrophages, manifested by the induction of platelet-derived growth factor, insulin-like growth factor-1, and proinflammatory cytokines such as IL-1β and TNF-α[82-84]. Similarly, the engagement of RAGE by S100A12 in cultured Bv2 cells (murine macrophages) induced production of IL-1β and TNF-α, in an NF-κB-dependent fashion[6]. S100B or AGEs treatment of THP-1 cells leads to a significant induction of COX-2 mRNA and protein[85]. Further, AGEs or S100A12 prompt monocyte migration (chemotaxis) mediated by the interaction with RAGE[86, 87].

AGEs may contribute to the expression of oxidized LDL (oxLDL) receptors in human monocyte–derived macrophages[88]. AGEs induce the gene expression of several OxLDL receptors such as macrophage scavenger receptor (class A and B), CD36 receptor, and lectin-like oxLDL receptor 1[88]. The increased expression of these receptors on macrophage membranes was closely associated with increased uptake of modified LDL, and culminated in enhanced foam cell transformation. Recently, it has been reported that in human macrophages the exposure to AGEs

reduced mRNA and protein levels in the ATP-binding cassette transporter G1[that promotes the efflux of cholesterol to larger high density lipoprotein (HDL) particles] in a RAGE-dependent manner[89]. In addition, functional studies demonstrated that exposure to AGEs decreased cholesterol efflux to HDL[89]. Thus, AGEs may be involved to foam cell formation via the increased numbers of OxLDL receptors[88] and via a decreased cholesterol efflux to HDL[89] in accelerated atherogenesis in diabetic patients (**Fig. 3**).

AGE-RAGE binding on SMC is associated with increased chemotactic migration and increased cellular proliferation[90] (**Fig. 3**). AGEs can contribute to extracellular matrix production by upregulation of transforming growth factor-β, a key regulator of extracellular matrix production by SMC[91]. In response to amphoterin, SMC proliferate, migrate, and secrete more amphoterin[60, 92]. Amphoterin-mediated migration of SMC results in activation of phosphorylated ERK 1/2 into the nucleus, but also involves a Gi/o protein[60].

Further in SMC, the ligand S100B increase the activation of Src kinase and tyrosine phosphorylation of caveolin-1 in a RAGE-dependent manner[93]. The Src tyrosine kinase inhibitor PP2, significantly blocks S100B-induced activation of Src kinase, MAPKs, NF-κB and STAT3, superoxide production, tyrosine phosphorylation of caveolin-1, SMC migration, and MCP-1/IL-6 gene expression [93].

Taken together, these studies underscore that some mechanisms by which the RAGE-ligand axis exerts its biological effects on vascular wall have been revealed. In summary, the RAGE-ligands might amplify inflammatory responses through enhanced generation of proinflammatory adhesion molecules, cytokines, and tissue-destructive matrix metalloproteinases. In the long term, the engagement of RAGE by these ligands may underlie continued amplification of

inflammatory events in tissues previously sensitised by lipid deposition or by immune/inflammatory triggers.

RAGE-ligand axis: lessons from animal models

In the last decade several animal models have been developed to dissect the contribution of RAGE-ligand interaction especially in the pathogenesis of diabetic vasculopathy. The impact of RAGE blockade (through the administration of the decoy protein sRAGE or anti-RAGE IgG) was first tested in an acute animal model of diabetes-associated hyperpermeability[94]. After 9-11 weeks, rats rendered diabetic with streptozotocin showed increased vascular permeability in multiple organs, that was normalized by RAGE blockade with either sRAGE or anti-RAGE IgG. Murine models of atherosclerosis have significantly advanced our understanding of the development of accelerated diabetic macrovascular disease. Since mice inherently resist the development of atherosclerosis, in part due to their high plasma levels of HDL, strains genetically susceptible to atherosclerosis have been used. In apolipoprotein (apo)E-null mice, which develop spontaneous atherosclerosis on a normal chow diet, streptozotocin-induced diabetes was associated with a significant increase of atherosclerotic lesion area and complexity at the aortic sinus after 6 weeks of diabetes compared with euglycemic apoE-null mice of the same age[40]. Diabetes-associated atherosclerotic lesions in this model featured increased AGE deposition and enhanced RAGE expression. Administration of sRAGE reduced the atherosclerotic lesion area, and lesion complexity [40]. Similarly, circulating AGEs, VCAM-1 and tissue factor vascular expression, and NF-κB induction all decreased in sRAGE-treated mice compared to vehicle-treated littermates. Interestingly, euglycemic animals receiving sRAGE also demonstrated a trend towards reduced atherosclerosis compared to vehicle-treated animals[40].

Another study in diabetic apoE-null mice sustains these observations also in established atherosclerotic lesions[95]. ApoE-null mice rendered diabetic with streptozotocin at the age of 6 weeks were left untreated until age 14 weeks. At this age, certain mice were sacrificed to establish the “baseline” area/complexity of atherosclerotic lesion. In those treated withsRAGE a stabilization of atherosclerotic lesion area and its complexity was observed[95]. In parallel RAGE blockade was associated with decreased vascular expression of VCAM-1, MCP-1, COX-2, nitrotyrosine epitopes, MMP-9 (antigen and activity), and tissue factor epitopes[95].

In a recent study, these concepts have been extended to a new murine model in which homozygous apoE-null mice were bred into the db/db background (db/db background results from genetic mutation of the leptin receptor and insulin resistance) to produce euglycemic and diabetic hyperlipidemic apoE-null m/db and apoE-null db/db mice, respectively[96].Compared to apoE-null m/db (non-diabetic) mice, apoE-null db/db (diabetic) mice displayed accelerated atherosclerosis at the aortic sinus. Administration of sRAGE, resulted in significantly reduced atherosclerotic lesion area in a glycemia- and lipid-independent manner[96]. In parallel, apoE-null db/db mice displayed RAGE-dependent enhanced expression of VCAM-1, tissue factor and MMP-9 antigen/activity in aorta compared to non-diabetic animals. In addition, consistent with the premise that upregulation of RAGE and its ligands occurs even in the non-diabetic, hyperlipidemic state, albeit to lesser degrees than in diabetes, administration of sRAGE to apoE-null m/db mice resulted in decreased atherosclerotic lesion area[96]. These findings highlight important roles for RAGE in proatherogenic mechanisms in hyperglycemia triggered by insulin resistance.

To clarify the biochemical and molecular events linked to enhanced restenosis in diabetic human subjects, diabetic rodent models of arterial injury have also been tested for the impact of diverse

interventions on neointimal expansion[97-99]. In diabetic rats undergoing carotid artery injury induced by balloon angioplasty, administration of sRAGE resulted in a significantly lower intima/media ratio versus that observed in diabetic rats treated with vehicle[98].

In another relevant study, wild-type mice, subjected to femoral arterial endothelial denudation, exhibited a prominent increase of RAGE and its ligands, CML and S100 proteins in the injured vessel wall, particularly in activated SMC[99]. Blockade of RAGE, employing sRAGE or antibodies, or in homozygous RAGE null mice, resulted in significantly decreased neointimal expansion after arterial injury and decreased SMC proliferation, migration, and expression of extracellular matrix proteins. A critical role for SMC RAGE signalling was demonstrated in mice bearing a transgene encoding a RAGE cytosolic tail-deletion mutant, specifically in SMC, driven by the SM22α promoter. Upon arterial injury, neointimal expansion was strikingly suppressed compared with that observed in wild-type littermates[99].

However, a more advanced system to test the role of SMC and monocyte RAGE in the response to acute arterial injury are apoE-null mice, because in these animals the basal vascular perturbation due to hyperlipidemia augment the influx of inflammatory cells into the injured vessel wall respect to wild-type animals. In fact, after 28 days of femoral artery injury, enhanced neointimal expansion in apoE-null mice vessels versus wild-type mice was observed. In euglycemic apoE-null mice, administration of sRAGE diminished intima/media ratio on day 28 and the amount of monocytes infiltrating the injured artery was also reduced[99]. Taken together, these data highlight key roles of RAGE in modulating SMC properties after injury, and they also suggest that RAGE is a valid target for suppression.

Although it has become evident that RAGE and its ligands, AGEs and S100 proteins, are critically implicated in the development of neointimal formation after balloon injury as well as in

atherosclerosis in animal models of diabetes, the mechanisms by which expression of RAGE is augmented in diabetes remain unclear. Takeda et al.[29] hypothesized that inflammatory cytokines could be implicated in the enhanced RAGE expression in the vessel wall and in neointima formation in a diabetic state. To block the actions of these endogenous cytokines they utilized two different strategies: one was a pharmacological inhibitor of proinflammatory cytokine production, the other was an adenovirus construct that expresses a dominant negative mutant of TNF receptor and blocks the signalling of TNF-α. Thus, they demonstrated that endogenous cytokines, especially TNF-α were implicated in the enhanced RAGE expression in the vessel wall and in neointimal formation in the femoral artery of obese Zucker rats[29].

RAGE functions also as an endothelial adhesion receptor promoting leukocyte recruitment by a direct interaction of RAGE with the leukocyte beta2-integrin Mac-1[8]. In fact, in mice with acute peritonitis induced by thioglycollate, leukocyte recruitment was significantly impaired in RAGE-null mice as opposed to wild-type mice, while in diabetic wild-type mice it was observed enhanced leukocyte recruitment to the inflamed peritoneum as compared with nondiabetic wild-type mice; this phenomenon was abrogated in the presence of sRAGE and was absent in diabetic RAGE-null mice[8]. The RAGE-Mac-1 interaction defines a novel pathway of leukocyte recruitment relevant in inflammatory disorders associated with increased RAGE expression.

Taken together, all studies employing RAGE-null mice have confirmed an important role for RAGE as a mediator of diabetic complications and macrovascular and chronic disease.

sRAGE: which role in the vascular disease?

RAGE expression, as above-described, increases in clinical settings characterized by enhanced cell activation and prolonged exposure of RAGE ligands and it determines a chronic state of cell

activation[48, 100, 101]. Interference with the vicious cycle established by RAGE-ligand interaction might interrupt cellular activation and consequently lead to an improvement of various chronic disorders[4, 7, 25]. As above mentioned, treatment with sRAGE dose-dependently suppresses the development of atherosclerosis in several animal models, acting as a RAGE competitor in ligand binding. For these important premises, in these last years, the C-truncated isoform of RAGE, that is secreted extracellularly and can therefore be detected in human sera[19] as endogenous secretory RAGE (esRAGE), has been object of intense clinical research.

Therefore, the decoy function of esRAGE suggests the presence of a regulatory negative feedback mechanism in which esRAGE can serve to prevent the activation of cell-surface RAGE and its harmful positive loop of regulation. However, it is possible that this secreted form alone does not correspond to the whole pool of sRAGE that exists in the bloodstream. Hence, it has been also suggested that a part of this pool may conceivably originate by proteolytical cleavage of the native membranous receptor via the action of extracellular metalloproteinases[102]. Recent clinical studies have investigate the potential meaning of serum sRAGE concentrations in several pathological conditions, particularly vascular disease and metabolic syndrome[103-109]. However, in these reports, the measure of circulating RAGE has been performed by using either antibodies generated generically to RAGE [103, 106-108] that cannot distinguish between the different splice variants of sRAGE as well as sRAGE cleaved from full-length receptor on cell surface) or antibodies which specifically detects esRAGE[104, 105, 109]. Although from these different evaluations, conflictual results are sometimes appeared, the molecular heterogeneity of the various soluble and cell-bound RAGE isoforms has deeply highlighted the complex regulation of the RAGE-ligand axis in several clinical settings, and a growing number of studies

suggests that the role of RAGE signalling is not mediated by the single action of anyone type of RAGE receptor, but is the summary of effects derived from each of the different RAGE isoforms.

The first study upon circulating sRAGE levels evidenced that, in age-matched Italian male subjects without diabetes, lower levels of plasma sRAGE were associated with enhanced risk of angiographically detected coronary artery disease[103]. Contemporaneously, it was reported that, in type 1 diabetic patients circulating esRAGE levels were significantly lower than in nondiabetic subjects and were inversely associated with the severity of some diabetic vascular complications[105]. Other clinical investigations examined plasma levels of sRAGE in relation to the components of the metabolic syndrome[104, 106, 109]. In one of these reports, the plasma levels of esRAGE were significantly lower in diabetic patients than nondiabetic controls, and were significantly and inversely correlated with components of the metabolic syndrome including body mass index, HbA1c, insulin resistance index, and with carotid or femoral atherosclerosis[104]. The same authors demonstrated in another recent report, that low circulating esRAGE levels are predictive for cardiovascular mortality in both nondiabetic and diabetic patients with end-stage renal diseases[109]. Accordingly, I and my colleagues have demonstrated that plasma sRAGE levels were lower in diabetic patients than controls and associated with glycemic control, proinflammatory factors, and insulin resistance index[106]. In addition in a subgroup of subjects we revealed that low sRAGE and high S100A12 plasma levels were strongly associated with increased risk for cardiovascular disease (Framingham score)[106]. However, some reports evidence increased, rather than decreased levels, of total sRAGE on type 2 diabetic patients [107, 108]. In one of these studies, circulating AGEs and sRAGE levels are associated with the severity of nephropathy in type 2 diabetic patients[107], while in another

study, serum sRAGE levels were significantly higher in type 2 diabetic patients than in non-diabetic subjects and positively associated with the presence of coronary artery disease[108].

It is likely that a compensatory anti-inflammatory phenomenon in response to tissue injury or decreased glomerular filtration rate could affect the total level of sRAGE in the bloodstream. Taken altogether these reports highlight that little is known about the regulation of endogenous sRAGE levels and further studies are needed to provide clarity about the biological mechanisms underlying the kinetics of sRAGE production and removal in health and disease states.

Conclusions and perspectives

The RAGE-ligand axis is a possible common etiological factor that contributes to multiple inflammation-based chronic diseases and could represent the relationship between environmental signal-induced inflammation and atherogenetic process. The biology of RAGE has extended beyond the original notion of a receptor for glycosylated proteins. The experimental evidence gathered thus far demonstrates unequivocally that ligand-RAGE interaction can alter vessel wall homeostasis in a pro-atherogenic fashion through multiple mechanisms, i.e., release of inflammatory cytokines, increased expression of adhesion molecules and chemokines, induction of SMC proliferation. Consequently, migration and activation of inflammatory cells that infiltrate the injured vessel wall sustain a process of chronic vascular inflammation. This progression of events thus prompts a vicious loop of enduring vascular injury, in part through the release of inflammatory molecules such as S100 proteins and amphoterin. Additionally, in diabetes, once inflammatory cells enter a basally perturbed tissue, inflammation wrought by these species triggers further oxidant stress and ongoing AGE generation, and this is a process that is difficult to end. Interference on the RAGE-ligand axis in these harmful processes may represent a valid

target for effective therapeutic interventions in two settings relevant to vascular biology, i.e., in innate chronic atherosclerosis as well as acute vascular injury.

The first studies were addressed to inhibit tissue accumulation of AGEs in diabetes, including inhibitors of AGE formation such as aminoguanidine or recognized cross-link breakers such as ALT 711[110-111]. In animal models, these inhibitors have potentially beneficial effects in reducing vascular diabetic complications[110-112]. Both aminoguanidine and AGE-crosslink breakers have been tested also in human and have revealed promising improvement in renal and in endothelial function [113, 114]. Alternative interventions by means of administration of sRAGE have been confined to studies on animal only [95, 96].

Some drugs currently in use for diabetic complications have shown to have an effect on AGE accumulation. Interestingly, the very used antidiabetic drug metformin (dimethylbiguanide) can prevents diabetic complications not only by lowering glycaemia, but also by inhibiting AGE formation and by activating antioxidant defences[115]. In summary, all approaches have been shown to confer some degree of antiatherosclerotic effects, although to different degrees and by different mechanisms.

In conclusion, although these premises will have to be further confirmed by clinical trials, they likely will provide a final word on the importance of this axis in vascular pathology – as well as the potential risks of its antagonism.

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Legend to the figures

Fig. 1 The structure of full–length RAGE and its splice variants

The V-type domain is critical for binding of RAGE-ligands. Deletion of this domain results in a N-truncated form that does not bind ligands. The C-truncated, circulating soluble RAGE contains only the extracellular domain of the receptor (V-C-C'). It may derive via enzymatic cleavage of full-length cell surface receptor and via release of several endogenous secretory splice variants of RAGE.

Fig. 2 Extracellular amphoterin mediates migration of cells, and inflammatory responses into the atherosclerotic plaque

After injury and necrosis, the endothelial cells (as most types of cells) passively release nuclear amphoterin into their surroundings. Otherwise, amphoterin can be actively secreted by monocyte-derived macrophages upon stimulation with TNF-α or IL-1. Extracellular amphoterin triggers in other RAGE-bearing cells a proper response for that cell type, like cell migration or inflammation.

Fig. 3 Implications for atherosclerosis of the vicious cycle RAGE-ligand axis

The AGE formation, as consequence of oxidative stress, hyperglycaemia, inflammatory stimuli, determine a site for the amplification of inflammatory pathways. In endothelial cell RAGE-ligand interaction increases leukocyte adhesion molecules expression and the tissue factor procoagulant activity. AGE-RAGE interaction triggers a vicious cycle of cellular injury, by upregulating the RAGE itself and by attracting polymorphonuclear leukocytes, monocytes and

lymphocytes. S100A12 protein and amphoterin release from such cells triggers a new wave of cell perturbing molecules. It promote the migration of circulating monocytes into the intima (chemotaxis), their conversion to activated macrophages, and their release of cytokines and proteases. Within the intima, activated macrophages increase their lipid uptake – mostly due to an increase of AGE-induced ox-LDL receptors – leading to the formation of foam cells. Next, activated SMC migrates into the intima at sites of vascular lesions, and here proliferates, producing new extracellular matrix.