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Biochimica et Biophysica Acta 1498 (2000) 99–111

BIOCHIMICA ET BIOPHYSICA ACTA

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## Review

# The biology of the receptor for advanced glycation end products and its ligands

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Received 11 September 2000; accepted 12 September 2000

## Abstract

Receptor for advanced glycation end products (RAGE) is a multiligand member of the immunoglobulin superfamily of cell surface molecules whose repertoire of ligands includes advanced glycation end products (AGEs), amyloid fibrils, amphoterins and S100/calgranulins. The overlapping distribution of these ligands and cells overexpressing RAGE results in sustained receptor expression which is magnified via the apparent capacity of ligands to upregulate the receptor. We hypothesize that RAGE-ligand interaction is a propagation factor in a range of chronic disorders, based on the enhanced accumulation of the ligands in diseased tissues. For example, increased levels of AGEs in diabetes and renal insufficiency, amyloid fibrils in Alzheimer's disease brain, amphoterin in tumors and S100/calgranulins at sites of inflammation have been identified. The engagement of RAGE by its ligands can be considered the 'first hit' in a two-stage model, in which the second phase of cellular perturbation is mediated by superimposed accumulation of modified lipoproteins (in atherosclerosis), invading bacterial pathogens, ischemic stress and other factors. Taken together, these 'two hits' eventuate in a cellular response with a propensity towards tissue destruction rather than resolution of the offending pathogenic stimulus. Experimental data are cited regarding this hypothesis, though further studies will be required, especially with selective low molecular weight inhibitors of RAGE and RAGE knockout mice, to obtain additional proof in support of our concept. © 2000 Elsevier Science B.V. All rights reserved.

**Keywords:** Diabetes; Inflammation; Amyloidosis; Tumor biology; Cell migration; Cell invasion

## 1. Introduction

The receptor for advanced glycation end products (RAGE) is a member of the immunoglobulin superfamily whose expression is upregulated at sites of diverse pathologies, from atherosclerosis to Alzheimer's disease [1]. One of the salient features of the receptor is its recognition of families of ligands, rather than a single polypeptide. A prime example

Abbreviations: A $\beta$ , amyloid- $\beta$  peptide; AGE, advanced glycation end product; CML, N<sup>c</sup>-(carboxymethyl)lysine; DN-RAGE, dominant-negative RAGE; DRA, dialysis-related amyloidosis; RAGE, receptor for AGE; M-CSF, macrophage-colony stimulating factor; sRAGE, soluble RAGE; TBIR, tissue-blood isotope ratio; VCAM-1, vascular cell adhesion molecule-1

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is the binding of S100/calgranulins to RAGE [2]. S100A12, also termed extracellular newly identified RAGE binding protein (EN-RAGE), was the first S100/calgranulin member shown to interact with RAGE in a dose-dependent and saturable manner resulting in activation of cellular targets. S100B derived from bovine brain was demonstrated to compete with S100A12 for binding to RAGE. Similarly, RAGE has been shown to interact with  $\beta$ -sheet fibrils composed of different subunits/monomers, amyloid A, amyloid- $\beta$  peptide, prion peptide, or amylin [3,4]. In each case, the most probable conclusion is that RAGE interacts with conformational determinants in the ligands allowing recognition of polypeptides with varied primary sequences. This indicates the importance of understanding the structure of ligand recognition sites in RAGE. X-Crystallography studies are in progress to accomplish this objective.

Several other features of RAGE biology are important to mention at the outset. First, there is an unusual sustained juxtaposition of ligand and receptor in tissues. At sites of accumulated advanced glycation end products (AGEs) in the vasculature, for example, there is increased expression of the receptor in cells of the vessel wall, including endothelium, vascular smooth muscle cells and invading mononuclear phagocytes [5]. Similarly, in amyloid-rich tissues, upregulation of RAGE is observed in parenchymal cells, such as neurons, as well as mononuclear phagocyte elements, including microglia in Alzheimer's disease brain [3]. Based on the apparently activated state of cells expressing RAGE at such loci (as reflected by sustained activation of NF- $\kappa$ B, expression of cytokines and cell adherence molecules, etc.), we speculate that the overlapping distribution of receptor and ligand results in prolonged receptor activation. Our studies have demonstrated that one facet of RAGE-mediated cellular stimulation includes increased expression of the receptor itself. Thus, one can envision a positive feedback loop in which ligand-receptor interaction increases expression of the receptor, thereby augmenting subsequent RAGE-induced cellular activation. This situation contrasts with other receptors, such as the LDL receptor, in which increased levels of ligand decrease expression of the receptor [6]. In fact, the only means we know to strongly downregulate RAGE is to in-

terrupt the cycle of ligand engagement of the receptor with soluble RAGE (see below) or blocking antibodies.

These considerations suggest that RAGE could function as a propagation factor in a number of disorders according to a two-hit model. The first hit is comprised of RAGE engagement by its ligands. Ligands such as AGEs and amyloid- $\beta$  peptide (A $\beta$ ) are present for long times in tissues (endogenous clearance mechanisms apparently do not function efficiently), and have the capacity to bring about sustained ligand-receptor interaction. The result of ligand engagement appears to be cellular activation, rather than clearance. In this context, RAGE-mediated endocytosis followed by lysosomal degradation is a slow process [7]. By contrast, the type A macrophage scavenger receptor is much more efficient when these two have been compared with respect to clearance of A $\beta$  [7]. The principal outcome of RAGE-ligand interaction is a change in the cellular set point resulting in a new basal state of activation. In the vasculature, this has been shown to include expression of vascular cell adhesion molecule-1 (VCAM-1) and induction of vascular hyperpermeability (i.e., increased vascular leakage) [8,9]. With a superimposed stimulus, such as accumulation of modified lipoproteins, cellular perturbation is further magnified. Rather than returning to homeostasis, cellular signal transduction mechanisms favor augmented dysfunction resulting in acceleration of atherogenesis (see below). The same logic can be applied to inflammatory/infectious stimuli, in which RAGE-induced activation of effector cells prevents their normal participation in protective and reparative processes.

In this brief review, the functional consequences of RAGE interaction with its different ligands will be summarized. These observations provide a context for considering the biology of this receptor in a spectrum of physiologically and pathophysiologically relevant situations.

## 2. AGEs and RAGE

Mechanisms of chronic vascular dysfunction have been much less studied than the acute response to proinflammatory cytokines. The transient induction

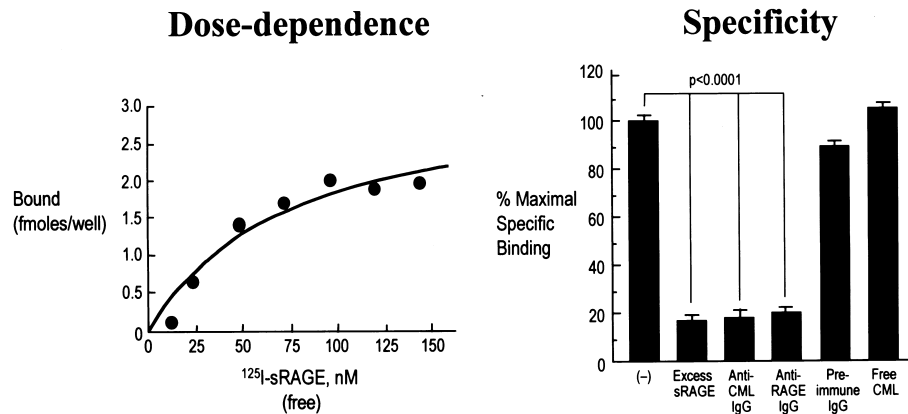


Fig. 1. Interaction of RAGE and CML-bovine serum albumin. Dose dependence: CML-modified bovine serum albumin (310 mmol CML per mol lysine) was immobilized in microtiter wells and a radioligand binding assay was performed with the indicated amount of  $^{125}\text{I}$ -sRAGE alone or in the presence of a 50-fold molar excess of unlabeled sRAGE. Specificity: the experiment was performed as in 'Dose dependence', except that  $^{125}\text{I}$ -sRAGE was added at 100 nM, and the indicated antibody was added (70  $\mu\text{g}/\text{ml}$  in each case). In each case, specific binding, defined as total (binding observed with  $^{125}\text{I}$ -sRAGE alone) minus nonspecific (binding observed with  $^{125}\text{I}$ -sRAGE in the presence of 50-fold excess unlabeled sRAGE) binding, is shown. Adapted from [16].

of leukocyte adherence molecules (especially in post-capillary venules), tissue factor (in certain vascular beds) and vascular hyperpermeability following exposure of endothelium to agents such as tumor necrosis factor- $\alpha$  and Interleukin 1 $\beta$  are hallmarks of the acute inflammatory response [10]. However, the situation is less defined in chronic vascular diseases such as diabetes. The inexorable accumulation of AGEs in the vessel wall provides a means of distinguishing normal from diabetic vessels [11–13]; i.e., the diabetic vessel has a memory based on its prolonged exposure to high glucose because of AGE modification of basement membrane-associated macromolecules. Such a site for deposition of AGEs allows their interaction with critical cells of the vessel wall, including endothelium and smooth muscle. The presence of AGEs in the vasculature has also been shown to promote trapping of macromolecules percolating through the vessel wall [11], and would be expected to affect cellular trafficking as well (for example, by engaging cells bearing RAGE on their surfaces). Furthermore, AGE cross-links change elasticity of the vessel wall [11]. Because of the close association of AGEs with cellular elements, it was logical to assess the molecular basis of AGE-cellular interactions. These considerations led us to isolate cell-associated polypeptides capable of mediating the interaction with AGEs. Since our initial goal was to focus on the vasculature, lung, with its rich

blood vessel network, provided an ideal starting point.

Our studies led to the isolation of a new member of the immunoglobulin superfamily which we named RAGE [14,15]. The extracellular domain includes three immunoglobulin-like regions; an N-terminal V-type domain followed by two C-type domains (termed C and C'). The principal ligand binding portion is the V-domain [16]. Though the C- and C'-domains may not directly bind ligands, they could have important roles in stabilizing the V-domain for mediating its interaction(s) with ligands. RAGE has a single transmembrane spanning domain and a 43 amino acid cytosolic tail which is highly charged. This cytosolic tail lacks known signaling motifs (phosphorylation sites, kinase domains, etc.), but is critical for RAGE-dependent cellular activation. A truncated form of RAGE from which the cytosolic tail has been deleted binds ligands identically to wild-type RAGE, though it does not mediate induction of cellular activation [2]. Cells expressing the latter variant of RAGE (termed tail-deletion, dominant-negative or DN-RAGE), even in the presence of wild-type RAGE, display inhibition of RAGE-induced signaling. This probably reflects a requirement for clustering of RAGE cytosolic tails, following engagement by the ligands, into a particular orientation which facilitates binding of cytosolic signaling complexes. The nature of the signaling molecules which bind

to the RAGE cytosolic tail is currently under intensive study.

The major AGE adduct of macromolecules found in vivo consists of *N*<sup>ε</sup>-(carboxymethyl)lysine (CML) [17]. We have found that CML-modified proteins (albumin, ovalbumin etc.) bind to RAGE [16]. The interaction of <sup>125</sup>I-soluble (s) RAGE with CML-modified bovine serum albumin is dose-dependent and saturable with  $K_d \approx 73$  nM (Fig. 1). Note that the interaction of <sup>125</sup>I-sRAGE with CML albumin was blocked by antibodies to RAGE or CML, as well as by excess unlabeled sRAGE (Fig. 1). There was no effect of preimmune IgG. Furthermore, CML alone was not a competitor, indicating that CML must be presented to the receptor in the appropriate context of a protein/peptide in order for receptor recognition to occur. Competitive binding experiments with purified RAGE domains showed that only excess unlabeled V-domain inhibited binding of <sup>125</sup>I-sRAGE to CML-albumin, not the C- or C'-domains [16]. Such CML adducts have the capacity to activate cells bearing RAGE resulting in nuclear translocation of NF- $\kappa$ B, induction of VCAM-1 expression and induction of chemotaxis. Consistent with this premise, following intravenous infusion of CML-bovine serum albumin into mice, expression of VCAM-1 was observed in extracts of lung by immunoblotting. VCAM-1 induction in this setting was blocked by anti-RAGE IgG or sRAGE (see below) indicating its dependence on RAGE-induced cellular activation. Preparations of AGE-modified polypeptides made in vitro often display about 30% modification of lysine residues which is a considerably greater degree of modification than occurs in vivo. The latter has been estimated to be in the range of 1.7–4.95 mmol of CML per mol of lysine based on studies of lens proteins and skin collagen obtained from elderly humans [18]. Thus, our studies of CML-induced RAGE activation have included preparations with as low as 0.8 mmol CML per mole of lysine [16]. Even with such preparations, RAGE-induced cellular activation was observed with CML-ovalbumin, but not with the native protein.

These data indicate that CML adducts of proteins are ligands for RAGE. The presence of low levels of CML in tissues provides a situation in which the basal state of activation of cells in the vasculature could be subtly changed over time. Since CML ac-

cumulation is accelerated by superimposed renal insufficiency and oxidant stress, especially that associated with inflammation, it is possible that properties of host effector cells are significantly altered in the AGE-rich environment via their interaction with RAGE.

These results led us to study the possible contributions of AGE-RAGE interaction in more complex biologic systems. In cell culture, experiments with AGEs, either those prepared in vitro or those derived from patients with diabetes or renal failure, demonstrated that consequences of AGE-cellular interactions appeared to result from engagement of RAGE. For example, AGE- $\beta_2$ -microglobulin isolated from the urine of patients with dialysis-related amyloidosis (DRA) induced chemotaxis and activation of mononuclear phagocytes [19]. Each of these effects was prevented by blockade of RAGE. Patients with DRA develop arthritis closely associated with deposition of AGE- $\beta_2$ -microglobulin in the joint space [20]. Macrophage activation is a prominent component of the resulting joint inflammation and eventual bone destruction. AGE interaction with RAGE on mononuclear phagocytes would provide a plausible mechanism for modulating cellular properties in this setting. Consistent with this hypothesis, immunohistochemical studies demonstrated the presence of RAGE-bearing cells in joints of patients with DRA which colocalized with the monocyte marker CD68 and displayed an overlapping distribution with AGE epitopes [19].

Red blood cells from patients with diabetes were found to bear cell surface AGEs capable of binding RAGE expressed by cultured endothelial cells [9,21]. The interaction of diabetic red cells with endothelium resulted in increased diffusional transit of tracers across the endothelial monolayer [9]. This change in properties of the endothelium was dependent on RAGE, as it was blocked by anti-RAGE IgG. At this point in our studies, it was clearly essential to move to in vivo model systems in order to determine if RAGE was a central receptor for AGE-induced cellular perturbation. For these studies it would be necessary to block RAGE for longer times, at least several months, and polyclonal anti-RAGE immunoglobulins would not be suitable reagents. As an alternative strategy, we prepared truncated form of RAGE, sRAGE, comprised of only the extracellular,

ligand binding domain [9]. In the presence of excess sRAGE, ligands bind to the soluble receptor and cellular activation is abrogated since the cell surface receptor remains unoccupied by ligands. Experiments in animal species and humans studied to date display low (picomolar) levels of sRAGE in the plasma, produced either by proteolytic processing or actual expression of truncated forms of the receptor. Thus, exogenous sRAGE of the same species evades immune surveillance mechanisms. Consistent with this observation, soluble RAGE has been administered to animals for over 6 months with no evidence of a local or systemic immune response.

Because of our results with diabetic red cells and the permeability of endothelial monolayers in vitro, our first in vivo studies assessed the AGE-RAGE axis in terms of its effect on vascular hyperpermeability in diabetic rats. Vascular hyperpermeability is a subtle and early hallmark of diabetic complications. Increased leakage of albumin in the urine at early stages in diabetes (microalbuminuria) is considered an important harbinger of later vascular complications [11]. Rats treated with streptozotocin became diabetic and were observed for 9–11 weeks (Fig. 2). Increased leakage of radiolabeled albumin was determined by the tissue-blood isotope ratio (TBIR; a ratio of  $^{125}\text{I}$ -albumin deposited to red cell

pooling in the indicated tissue, the latter based on accumulation of  $^{51}\text{Cr}$ -labeled erythrocytes) [9,22]. Using this assay, increased vascular leakage of albumin was present in a range of tissues from the diabetic animals, compared with untreated controls. Animals receiving a single treatment with sRAGE displayed marked suppression of vascular hyperpermeability. This indicated that vascular dysfunction, as manifested by increased leakiness of the blood vessels, was reversible at this stage of diabetes, and that RAGE-dependent mechanisms were likely involved.

Diabetic macrovascular disease is a major cause of morbidity and mortality, and is not equally responsive to rigorous glucose control compared with microvascular disease. These considerations led us to assess the effect of RAGE blockade in a murine model of accelerated atherosclerosis-diabetes [23]. ApoE null mice rendered diabetic with streptozotocin develop atherosclerosis at an accelerated rate compared with euglycemic controls; lesions develop more rapidly, are greater in number, cover a greater area of the vessel surface and display increased complexity (presence of cholesterol clefts, necrosis and/or fibrous caps) compared with the same time point in nondiabetic apoE null mice. Using this model, mice were treated with either sRAGE or vehicle (mouse

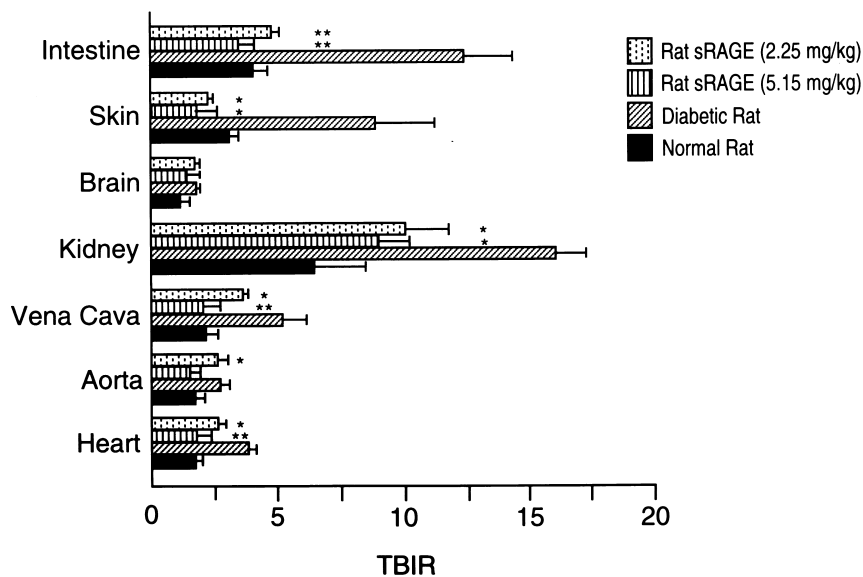


Fig. 2. Administration of sRAGE decreases vascular hyperpermeability in diabetic rats. Diabetic rats (induced with streptozotocin and observed for 9–11 weeks) were infused with sRAGE at the indicated dose (intraperitoneally) and then tissue-blood isotope ratio was determined to assess vascular permeability. Adapted from [9].

serum albumin), and lesion formation was assessed 6 weeks later. The sRAGE-treated apoE null diabetic mice showed strong inhibition of lesion formation; there were fewer lesions which occupied less area in the sRAGE-treated group compared with those animals receiving vehicle alone. The lesions which were present in sRAGE-treated diabetic apoE null mice appeared to be arrested at an earlier stage; i.e., they were predominately fatty streaks compared with a much larger percentage of complex lesions in vehicle-treated apoE null diabetic mice. These results have been extended to LDL receptor null and transgenic apoB mice, and to the db/db strain (the latter providing a genetic model of insulin resistance, thus obviating the need for streptozotocin) with similar results.

We have hypothesized that sRAGE inhibition of lesion formation in atherosclerosis-prone diabetic mice is due to interception of RAGE-ligand interactions. There is no change in the level of glycemia or hemoglobin A<sub>1c</sub> in mice treated with sRAGE [23]. Furthermore, total cholesterol, which rises on induction of diabetes, and lipoprotein profile, remain unaffected by administration of sRAGE. However, animals treated with sRAGE displayed strikingly decreased AGE content in the vasculature and tissues. Furthermore, complexes of sRAGE bound to AGE-immunoreactive species could be detected in the plasma of treated animals, consistent with their interaction *in vivo*. In terms of mechanisms through which sRAGE prevents AGE accumulation in tissues, both increased clearance (possibly reflected by the presence of sRAGE-AGE complex in the plasma) and decreased formation (the latter may be due to suppression of oxidant stress, a part of the RAGE-induced signaling cascade) are possible. These data highlight a lipid- and glycemia-independent means through which atherogenesis is accelerated in diabetic animals. According to these models, the activation of RAGE appears to have a central role in accelerating atherosclerosis and leading to the formation of complex lesions with infiltrating mononuclear phagocytes and smooth muscle cells.

Our subsequent studies have addressed the issue as to whether RAGE blockade might impact on other complications in diabetes. Many of these studies are underway, but early results in nephropathy and periodontal disease have suggested a striking beneficial

effect of blocking RAGE. One important consideration in these studies is the possibility that more than one RAGE ligand may be present in tissues at the same time. This is discussed further below, and indicates the potential for several ligands to reinforce RAGE-dependent cellular activation.

### 3. Amyloid fibrils and RAGE

Another family of disorders whose manifestations include chronic vasculopathy include the amyloidoses. Cerebral vascular amyloid angiopathy, in which amyloid deposits in the basement membrane of cerebral blood vessels, is often associated with Alzheimer's disease [24]. Patients with the Dutch-type variant of A $\beta$  have the most striking evidence of vascular amyloid accumulation; in fact, the mutant amyloid preferentially deposits in the vessels, to a much greater degree than that in cerebral parenchyma, and results in hemorrhagic strokes [25]. In view of the many effects of A $\beta$  on cellular elements of the vessel wall, as well as in the cerebral parenchyma itself, we began an intensive search to identify cellular interaction sites for A $\beta$ . To our surprise, once again we identified RAGE as a cell surface receptor capable of mediating A $\beta$ -induced cellular perturbation [3]. It is important to note that RAGE binds A $\beta$  in the nanomolar range (50–150 nM), and that at these levels, we have found RAGE-bearing cells to display increased susceptibility to the effects of A $\beta$ . The type of A $\beta$ -RAGE-mediated changes in cellular properties that we have observed include expression of macrophage-colony stimulating factor (M-CSF) [26], activation of NF- $\kappa$ B [3], activation of caspase 3, and induction of DNA fragmentation. Whereas neurons undergo RAGE-dependent apoptosis after prolonged incubation with A $\beta$  fragments, microglia demonstrate sustained cellular activation; the latter includes expression of cytokines and M-CSF, and chemotaxis. Each of these effects is blocked by anti-RAGE IgG or in the presence of excess sRAGE, indicating the key role of RAGE. However, it is important to note that the outcome of RAGE ligation on these two cell types is different. The reasons for these differences are not yet clear, but probably relate to the specialized properties of neurons versus microglia. For example, although neurons elaborate M-CSF in re-

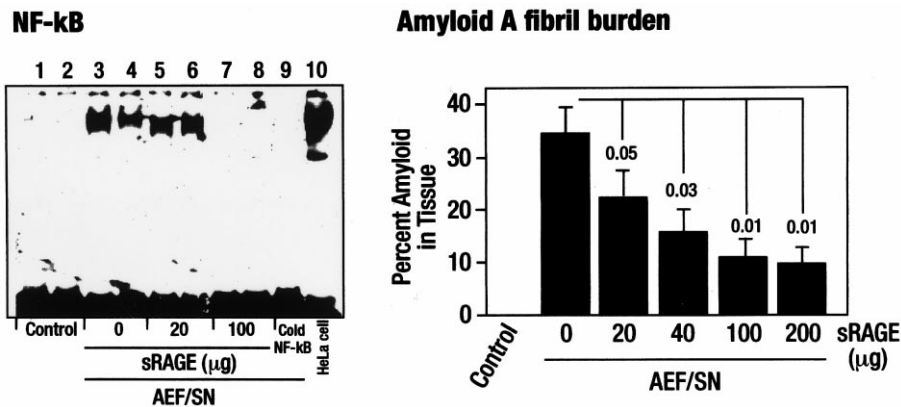


Fig. 3. In a model of systemic amyloidosis, administration of sRAGE suppresses splenic NF- $\kappa$ B activation and accumulation of amyloid A. Animals were treated with amyloid enhancing factor/silver nitrate either alone, or with daily administration (intraperitoneally) with the indicated concentration of sRAGE. Animals were sacrificed by humane euthanasia and splenic tissue was obtained to prepare nuclear extracts for NF- $\kappa$ B or for assessment of amyloid A fibril burden (the number above the bar showing amyloid A burden indicates the *P* value). The electrophoretic mobility shift assay was performed with a consensus  $^{32}$ P-labeled NF- $\kappa$ B probe. 'Cold' indicates a lane in which excess unlabeled NF- $\kappa$ B probe was added. Nuclear extracts from HeLa cells were added as a positive control. Adapted from [30].

sponse to incubation with A $\beta$ , they are unable to respond this mediator, since they do not express the receptor, c-fms [26]. In contrast, microglia express c-fms and, thus, can respond to M-CSF present in Alzheimer's disease brain. M-CSF promotes cell survival, and even proliferation, in the presence of A $\beta$ , and may prove to be a key nutritive factor for microglia in the harsh A $\beta$ -rich environment. We propose a scheme in which neuronal-derived M-CSF draws microglia to sites of A $\beta$  deposition at early stages in Alzheimer's disease. Microglia flourish in this milieu, and products of their activation further damage neurons. The increased levels of M-CSF in Alzheimer's disease brain and cerebrospinal fluid are consistent with this hypothesis [26], though further experiments will be required to fully verify our concept. An important consideration in these studies is to compare receptor-dependent and receptor-independent events related to the interaction of A $\beta$  with cells. At high concentrations of A $\beta$  (10–50  $\mu$ M), receptor-dependent events are overwhelmed by receptor-independent phenomena, such as A $\beta$ -induced formation of oxygen free radicals and direct A $\beta$  destabilization of membranes [27–29]. Thus, at these concentrations, blocking RAGE has no effect on the toxic effects of A $\beta$  on cells. However, at nanomolar levels of A $\beta$ , receptor-dependent phenomena predominate (i.e., blocking RAGE prevents cellular effects of A $\beta$ ). The clear question concerns which of

these situations is relevant in vivo. One possible scenario is that lower concentrations of A $\beta$  earlier in Alzheimer's disease render receptor-dependent mechanisms of cellular perturbation more relevant at these times. In contrast, later in the course of Alzheimer's disease, when levels of A $\beta$  are much higher, nonspecific (receptor-independent) cytotoxicity might become the predominant mode of cellular perturbation. Studies are in progress in transgenic models to directly test the possible role of RAGE in cellular perturbation underlying Alzheimer-type pathology.

In order to more directly test the consequences of RAGE serving as a receptor for amyloid, we also performed experiments with fibrils comprised of amylin, amyloid A, and prion-derived peptide [30]. These experiments showed that only the fibrillar form of these peptides bound to RAGE (binding of immobilized fibrils to the receptor was dose-dependent and displayed  $K_d \approx 50$ –150 nM, as with the AGE ligands and A $\beta$  described above). When presented to the receptor in random conformation, none of these peptides bound RAGE. Similarly, RAGE did not bind collagen or elastin fibrils. Furthermore, erabutoxin B, a protein with high  $\beta$ -sheet content, but which does not form fibrils, did not bind to RAGE. These data suggested that RAGE could serve as a cell surface receptor for  $\beta$ -sheet fibrils. In vitro studies demonstrated RAGE-dependent cellular activation in cultured BV-2 microglial cells exposed

to each of these amyloid fibrils. Because of the availability of models of systemic amyloidosis [31,32], as well as the accessibility of the amyloid-cellular interaction to inhibition by sRAGE or anti-RAGE F(ab')<sub>2</sub> (i.e., amyloid deposition occurs in areas accessible to the systemic circulation, rather than behind the blood-brain barrier as it does in models of Alzheimer-type pathology), we were able to carefully examine the role of RAGE in cellular perturbation and amyloid deposition [30]. Treatment of mice with amyloid enhancing factor/silver nitrate was used to induce deposition of amyloid A in the spleen. Accumulation of splenic amyloid was closely correlated with expression of cell stress markers, such as heme oxygenase type 1, Interleukin 6, and M-CSF, as well as activation of NF- $\kappa$ B (Fig. 3, NF- $\kappa$ B). Analysis of RAGE expression demonstrated that levels of the receptor increased in the spleen, and its distribution paralleled that for deposition of the amyloid. These data strongly suggested the possibility that RAGE-amyloid A might interact *in vivo*. Thus, we performed experiments in the presence of sRAGE or anti-RAGE F(ab')<sub>2</sub> to determine if blocking access of ligands to the receptor might affect cellular perturbation or amyloid deposition, focusing on the spleen. Animals receiving sRAGE or anti-RAGE F(ab')<sub>2</sub> displayed comparable elevation of plasma serum amyloid A, compared with those treated with vehicle. However, RAGE blockade suppressed evidence of cellular perturbation based on inhibition of heme oxygenase type I, M-CSF and Interleukin 6 expression and NF- $\kappa$ B activation (Fig. 3, NF- $\kappa$ B). Furthermore, amyloid deposition in the spleen was also suppressed by RAGE blockade (Fig. 3, amyloid A fibril burden). These experiments also provided direct evidence of amyloid A-RAGE interaction; immunoprecipitation of plasma from mice treated with sRAGE demonstrated complexes of amyloid A and sRAGE.

These studies demonstrate that RAGE binds amyloid fibrils and mediates cellular responses in the nanomolar range. Studies in genetically manipulated mice will be necessary to directly prove the role of RAGE, especially experiments with RAGE null mice and animals overexpressing wild-type receptor. These mice can then be crossed with models of amyloid pathology, such as mice overexpressing transgenes including mutant forms of amyloid precursor protein

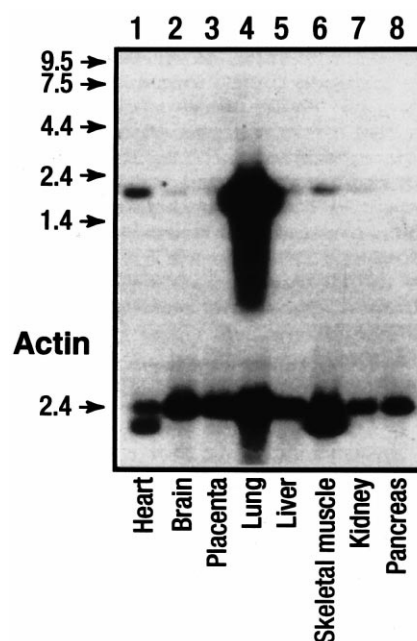


Fig. 4. Northern analysis of RAGE and  $\beta$ -actin transcripts in normal human tissues. Adapted from [33].

associated with deposition of A $\beta$  in the brain. Such studies are an important focus of our current work on RAGE.

#### 4. S100/calgranulins and RAGE

The interaction of RAGE with AGEs and amyloid fibrils seemed most likely to be fortuitous. Neither of these structures is present under physiologic conditions, and, thus, we sought a ligand for the receptor in normal tissues. Northern analysis of RAGE in normal human tissues displayed highest levels of RAGE transcripts in the lung (Fig. 4) [15,33]. Further studies showed lung to contain the greatest amount of RAGE antigen, leading us to begin the search for natural ligands of RAGE in this tissue. The extracellular domain of RAGE was immobilized on affinity columns, and protein extracts of lung based on their ability to bind RAGE. Two polypeptides were isolated using this purification and assay system [34]. The first was a member of the amphoterin family (see below) and the second was a member of the S100/calgranulin family, EN-RAGE or S100A12. As mentioned above, RAGE not only bound S100A12/EN-RAGE, but also interacted



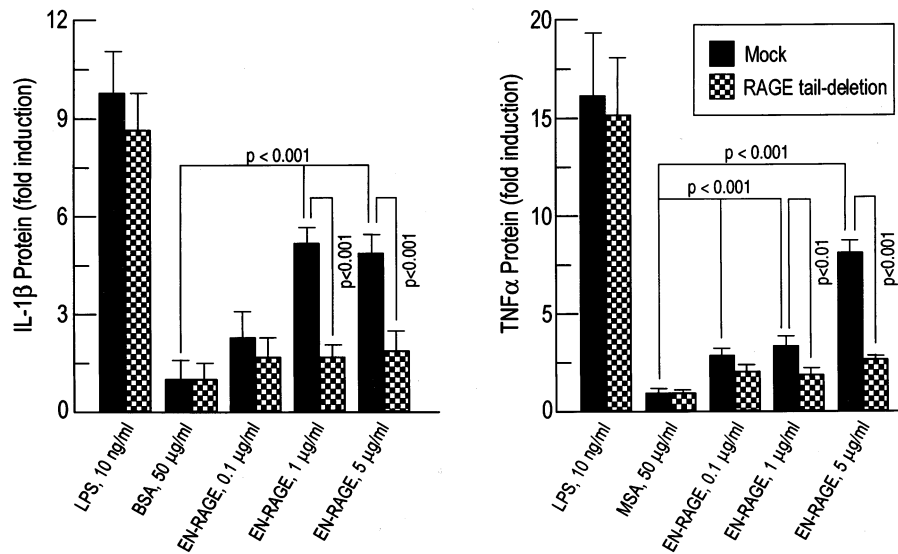


Fig. 5. Induction of cytokines (IL-1 $\beta$ , and TNF- $\alpha$  antigens) by transformed murine microglial cells (BV-2 cells) exposed to lipopolysaccharide (LPS), bovine serum albumin (BSA) or EN-RAGE/S100A12 (at the indicated concentration). BV-2 cells were either mock-transfected or transfected to overexpress dominant-negative (DN)-RAGE. Adapted from [2].

with S100B [2]. This has led us to suggest that RAGE may engage a spectrum of S100/calgranulins with the interaction mediated by common structural determinants in the ligands.

Incubation of S100A12/EN-RAGE with transformed murine macrophages (BV-2 cells) resulted in elaboration of Interleukin 1 $\beta$  and tumor necrosis factor- $\alpha$  (Fig. 5) [2]. In each case, transfection of the cells to express dominant-negative (DN; also termed tail-deleted receptor)-RAGE prevented expression of the cytokines, even in the presence of wild-type RAGE on the BV-2 cells (Fig. 5). Thus, DN-RAGE does function as a dominant negative for blocking the effects of RAGE-dependent cellular activation. This experiment, along with studies with anti-RAGE IgG/F(ab')<sub>2</sub> and sRAGE have shown that RAGE is an important cellular interaction site for S100/calgranulins. The recognition that a single receptor might mediate the effect of multiple S100/calgranulin family members in vivo posed easily testable questions: would RAGE mediate the effect of S100/calgranulins in delayed-type hypersensitivity and other inflammatory disorders? Our strategy was to block RAGE using anti-RAGE F(ab')<sub>2</sub> and sRAGE in animal models of inflammation.

Our first experiments employed a murine model of delayed-type hypersensitivity in which methylated bovine serum albumin was used as the preparatory

and provocative agent [2]. Severe edema and inflammation was evident in the footpad, along with activation of the transcription factor NF- $\kappa$ B and expression of Interleukin 2 and tumor necrosis factor- $\alpha$  transcripts. Animals treated with anti-RAGE F(ab')<sub>2</sub> or sRAGE showed striking suppression of the inflammatory response. Furthermore, blockade of RAGE also inhibited nuclear translocation of NF- $\kappa$ B, as well as expression of Interleukin 2 and tumor necrosis factor- $\alpha$ , indicating that preventing ligand access to the receptor impacted on the inflammatory cascade at a very proximal step. These initial results were extended to a murine model of colitis in Interleukin 10 null mice [35]. These mice have been shown to develop colitis, associated with activation of NF- $\kappa$ B in colonic tissue and systemic expression of tumor necrosis factor- $\alpha$  [2]. Administration of sRAGE had a strong protective effect mitigating the locally destructive inflammatory response, as well as plasma levels of tumor necrosis factor- $\alpha$ .

In the case of the delayed-type hypersensitivity model, anti-S100A12/EN-RAGE F(ab')<sub>2</sub> fragments were also delivered to the animals [2]. This had an inhibitory effect, though not as potently as blocking RAGE. Experiments using anti-S100A12/EN-RAGE and anti-RAGE F(ab')<sub>2</sub> showed a marked suppression of inflammation. Since our anti-S100A12/EN-RAGE antibody is polyclonal and raised to the in-

tact protein, it is not selective for only this S100/calgranulin family member. In fact, it is likely to display immunoreactivity with many family members. Thus, we believe that our results support a role for S100/calgranulins interacting with RAGE as an important component of an evolving inflammatory response. However, it is difficult to rule out contributions of other RAGE ligands, such as CML adducts which are also likely to form in the inflammatory milieu. The identification of RAGE as a cell surface target of S100/calgranulins provides a means to test the contribution of this interaction in a range of settings based on blockade of RAGE. This is technically simpler than blocking or suppressing expressing the large S100/calgranulin family.

## 5. Amphotericins and RAGE

Amphotericins are part of the family of high mobility group 1 nonhistone chromosomal DNA binding proteins [36,37]. Although their function in gene regulation/structure is not known, they have been shown to exert effects following their release from cells. *In vitro* studies have shown that amphotericin promotes neurite outgrowth and provides a site for the assembly of enzyme/substrate complexes leading to the generation of plasmin [36,37]. Amphotericin expression is also associated with tumors, and recent studies have shown amphotericin to be a late mediator of endotoxin toxicity *in vivo* [38]. Although these data might appear to comprise an apparently disconnected set of observations, it seemed possible that RAGE, via its interaction with amphotericin, could provide a basis for drawing together at least some of these effects.

First, we demonstrated that RAGE bound amphotericin selectively and in a saturable manner [34]. Based on these observations, studies were performed with primary cultures of rat cortical neurons plated on amphotericin-coated substrates. Neurite outgrowth was found to depend on RAGE-amphotericin interaction. Subsequently, this observation was extended by the laboratory of Dr. Heikki Rauvala (University of Helsinki) by their demonstration that expression of dominant-negative RAGE blocked the effect of amphotericin, in terms of induction of neurite outgrowth, and triggering of a signaling pathway involving Rac

and cdc42 [39]. The presence of RAGE at the leading edge of neurites, where it could engage amphotericin, suggested the potential for the receptor to mediate cell migration. Furthermore, if amphotericin anchored to cell surface RAGE provided a site for plasmin generation, this would further favor an invasive cellular phenotype. These considerations led us to analyze roles for RAGE in the migration and invasion of tumor cells.

The possible relevance of RAGE to tumor biology was underscored by immunoblotting studies showing higher levels of RAGE in many tumors compared with the normal tissue counterpart (breast, colon, kidney, stomach, etc.) [40]. For these studies, two experimental tumor models were employed, local/primary tumors (rat C6 glioma cells) and metastasis (Lewis Lung model). In each case, the tumor cells were found to express both RAGE and amphotericin. First, primary tumors were raised in immunocompromised mice following injection of wild-type C6 glioma cells [40]. Tumor volume was diminished in the presence of anti-RAGE F(ab')<sub>2</sub> or sRAGE, but there was no effect of nonimmune F(ab')<sub>2</sub>. To further analyze the role of RAGE, stably transfected lines of C6 glioma cells were made overexpressing either wild-type RAGE, DN-RAGE, sRAGE or vector alone [40]. When tumors were raised following local injection of these clones, there was a dramatic difference in the volume (Fig. 6) and histologic appearance of the resulting neoplasms which formed. Whereas tumors overexpressing wild-type receptor were very large, compared with vector controls, tumor volume was markedly diminished in tumors comprised of cells in which RAGE was blocked (tumors comprised of C6 glioma cells overexpressing DN-RAGE or sRAGE). Pathologic studies showed that blockade of RAGE correlated with the formation of small, well-circumscribed tumors during the experimental period. In contrast, tumors overexpressing wild-type RAGE grew rapidly and invaded the surrounding tissue very efficiently.

These observations were next extended to the Lewis Lung tumor model of metastasis [40]. Following resection of the primary tumor, lung metastases were compared in mice treated with sRAGE or vehicle alone. There was a striking decrease in the number of surface lung metastases observed in mice receiving sRAGE. These data in both primary and

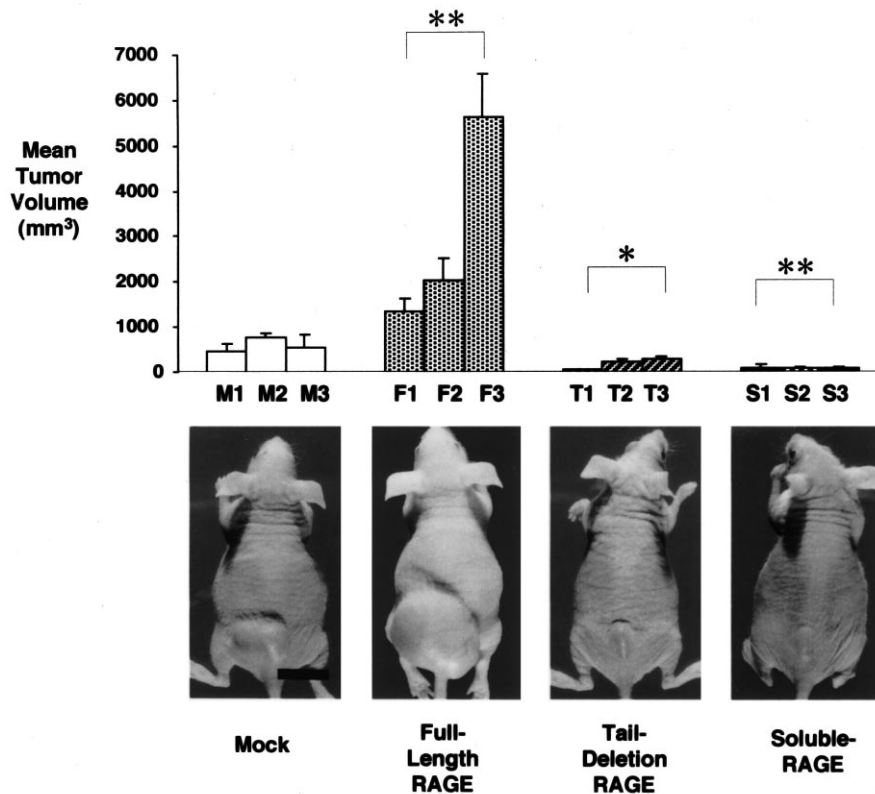


Fig. 6. Tumors were raised in immunocompromised mice by injecting either mock-transfected C6 glioma cells or C6 glioma cells stably transfected to overexpress full-length RAGE, tail-deletion (or dominant-negative) RAGE or sRAGE. Tumors were allowed to grow, and then animals were sacrificed and tumor volume determined. Adapted from [40].

metastatic tumor models led us to probe mechanisms underlying the protective effect of sRAGE. C6 glioma cells overexpressing wild-type RAGE plated on amphotericin-coated matrices displayed strong activation of mitogen-activated protein kinases, including p38, SAPK (stress-activated protein kinase) and Erk (extracellular regulated kinase) 1/2. Downstream effects of these and other signaling mechanisms triggered by RAGE-ligand interaction led to increased cell migration and expression of matrix metalloproteinases. One likely consequence of these RAGE-induced changes in cellular properties was the increased invasiveness of C6 glioma cells overexpressing RAGE in assays using Matrigel. These data are consistent with our *in vivo* observations that following implantation of tumor cells, those in which RAGE was blocked (by overexpression of DN-RAGE or sRAGE) displayed a delay in cell migration and proliferation (without an associated change in the apoptotic index). We propose that RAGE blockade induces a type of transient dor-

mancy of the tumor cells. In contrast, there did not appear to be an effect of RAGE expression on tumor vasculature. These data suggest the possibility that inhibitors of RAGE might be combined with agents with anti-angiogenic and direct tumor cell cytotoxic activity for a maximal effect on tumor properties. In addition, it is important to mention that in contrast to murine tumors, in which cell proliferation and angiogenesis must occur over days for tumor survival and growth, human tumors have a much more protracted time course, over years (especially in the initial phases). Because of this consideration, we are optimistic that blockade of RAGE may prove to be an effective adjunct to currently envisioned therapies.

## 6. Conclusion/hypotheses

The multiligand character of RAGE allows it to participate in an apparently wide spectrum of pathologic events. A common denominator of these path-

ologies is the role of RAGE as a propagator of cellular dysfunction. This occurs due to ligand induction of the receptor, sustained ligand-induced cellular activation, and the presence of RAGE ligands in the tissues for long times. Thus, in atherosclerotic vasculature, the presence of both AGEs and S100/calgranulins reinforces RAGE-dependent cellular activation. In Alzheimer's disease, the presence of A $\beta$  fibrils occurs along with S100 proteins again results in the presence of two RAGE ligands. In inflammatory settings, S100/calgranulins, AGEs, and, potentially, amphotericin could be present at lesional sites.

RAGE triggers a cascade of signaling mechanisms whose analysis has just begun. Activation of p21<sup>ras</sup> and phosphatidylinositol 3-kinase recruits pathways involving mitogen-activated protein kinases [41,42]. In addition, Rho family small GTPases are activated, especially cdc42 and Rac, potentially important in RAGE-induced changes in cell migration [39]. The generation of reactive oxygen species, an early event after ligation of the receptor, may be fundamental for many RAGE-induced changes in cellular properties. The important point is that from the myriad of pathways recruited by RAGE-induced cellular activation, it is virtually impossible, a priori, to predict with certainty the outcome of RAGE-dependent activation in a particular cell type. Thus, in the case of mononuclear phagocytes, RAGE-dependent stimulation results in activated phenotype with increased chemotaxis and release of proinflammatory cytokines. In contrast, in the case of neurons, RAGE-induced cellular activation ultimately results in induction of programmed cell death.

Many studies remain to be done concerning the contribution of RAGE to inflammatory disorders, diabetic complications and amyloidoses. The reagents for these studies, including means to block RAGE and genetically manipulated mice (knockouts and transgenics overexpressing wild-type and dominant negative RAGE), are becoming available. Thus, an understanding of RAGE in the broader context of the biology of host response mechanisms should be possible in the coming years.

#### Acknowledgements

This work was supported by grants from the

USPHS, a grant from the Juvenile Diabetes Foundation, and the Surgical Research Fund.

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