The receptor for advanced glycation end products (RAGE) is a central mediator of the interaction of AGE-beta2microglobulin with human mononuclear phagocytes via an oxidant-sensitive pathway. Implications for the pathogenesis of dialysis-related amyloidosis.

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An important component of amyloid fibrils in dialysis-related amyloidosis is a form of beta2microglobulin modified with advanced glycation end products (AGEs) of the Maillard reaction, known as AGE-beta2M. We demonstrate here that the interaction of AGE-beta2M with mononuclear phagocytes (MPs), cells important in the pathogenesis of the inflammatory arthropathy of dialysis-related amyloidosis, is mediated by the receptor for AGEs, or RAGE. 125I-AGE-beta2M bound to immobilized RAGE or to MPs in a specific, dose-dependent manner (Kd approximately 53.5 and approximately 81.6 nM, respectively), a process inhibited in the presence of RAGE blockade. AGE-beta2M-mediated monocyte chemotaxis was prevented by excess sRAGE or anti-RAGE IgG. Induction of tumor necrosis factor-alpha (TNF) expression by MPs exposed to AGE-beta2M resulted from engagement of RAGE, as appearances of TNF transcripts and TNF antigen release into culture supernatants were prevented by addition of sRAGE, a process mediated, at least in part, by oxidant stress. AGE-beta2M reduced cytochrome c and the elaboration of TNF by MPs was inhibited by N-acetylcysteine. Consistent with these data, immunohistochemical studies of AGE-laden amyloid deposits of a long-term hemodialysis patient revealed positive staining for RAGE in the MPs infiltrating these lesions. These data indicate that RAGE is a central binding site for AGEs formed in vivo and suggest that AGE-beta2M-MP-RAGE interaction likely contributes to the initiation of an inflammatory response in amyloid deposits.
The Receptor for Advanced Glycation End Products (RAGE) Is a Central Mediator of the Interaction of AGE-β2-Microglobulin with Human Mononuclear Phagocytes Via an Oxidant-sensitive Pathway

Implications for the Pathogenesis of Dialysis-related Amyloidosis

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Abstract

An important component of amyloid fibrils in dialysis-related amyloidosis is a form of β2-microglobulin modified with advanced glycation end products (AGEs) of the Maillard reaction, known as AGE-β2M. We demonstrate here that the interaction of AGE-β2M with mononuclear phagocytes (MPs), cells important in the pathogenesis of the inflammatory arthropathy of dialysis-related amyloidosis, is mediated by the receptor for AGEs, or RAGE. 125I-AGE-β2M bound to immobilized RAGE or to MPs in a specific, dose-dependent manner (Kd ≈ 53.5 and ≈ 81.6 nM, respectively), a process inhibited in the presence of RAGE blockade. AGE-β2M-mediated monocyte chemotaxis was prevented by excess sRAGE or anti-RAGE IgG. Induction of tumor necrosis factor-α (TNF) expression by MPs exposed to AGE-β2M resulted from engagement of RAGE, as appearances of TNF transcripts and TNF antigen release into culture supernatants were prevented by addition of sRAGE, a process mediated, at least in part, by oxidant stress. AGE-β2M reduced cytochrome c and the elaboration of TNF by MPs was inhibited by N-acetylcysteine. Consistent with these data, immunohistochemical studies of AGE-laden amyloid deposits of a long-term hemodialysis patient revealed positive staining for RAGE in the MPs infiltrating these lesions. These data indicate that RAGE is a central binding site for AGEs formed in vivo and suggest that AGE-β2M-MP-RAGE interaction likely contributes to the initiation of an inflammatory response in amyloid deposits of long-term hemodialysis patients, a process which may ultimately lead to bone and joint destruction. (J. Clin. Invest. 1996, 98:1088–1094.) Key words: glycated proteins • cytokines • long-term hemodialysis patients • monocyte • amyloidosis

Introduction

Proteins or lipids exposed to aldose sugars undergo nonenzymatic glycation and oxidation (1–7). Initially, the reversible early glycation adducts form, the best known of which is hemoglobin A1c, used as an index of glycemic control in diabetic patients. After further complex molecular rearrangements, the irreversible advanced glycation end products (AGEs) form. Conditions favoring AGE formation include those in which protein/lipid turnover is prolonged or delayed on lysine-rich structures, especially in the setting of elevated levels of aldose sugars, such as in diabetes. AGE formation can occur even in the euglycemic state, as in hypercholesterolemic rabbits without diabetes, in which macromolecules are trapped in the expanded neointima in atherosclerosis-prone animals (8).

While the generation of AGEs is particularly likely in the diabetic milieu given relatively sustained hyperglycemia (1–4), the tendency toward generation of AGEs is also favored on long-lived proteins, such as those central in the pathogenesis of amyloidoses. In Alzheimer’s disease, AGE modification occurs on components of the intracellular neurofibrillary tangles and in extracellular amyloid-β peptide (9–11). In a different context, renal failure presents a unique pathophysiological setting for the development of AGE formation on certain proteins which otherwise would not be susceptible to such irreversible modification, such as those that normally circulate in the plasma for limited periods of time, the handling of which is largely controlled by functioning kidneys. Our previous work has identified the presence of an irreversibly glycated form of β2M in the amyloid fibrils of long-term hemodialysis patients who develop dialysis-related amyloidosis (DRA) (12, 13). As the interaction of AGEs with cellular elements is a principal mechanism through which these glycated adducts exert their biologic effects (14–18), we sought to characterize in molecular terms AGE-β2M-mediated perturbation of mononuclear phagocytes (MPs), the latter believed to have a pivotal role in the pathogenesis of DRA. We demonstrate here that AGE engagement of the monocyte receptor for AGE, or RAGE, a cell surface polypeptide of the immunoglobulin superfamily (14, 19), is a first step in AGE-β2M-mediated activation of MPs. That is, RAGE mediates the binding of AGE-β2M to the cell

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1. Abbreviations used in this paper: AGE, advanced glycation end product; AGE-β2M, AGE-modified β2-microglobulin; DRA, dialysis-related amyloidosis; MPs, mononuclear phagocytes; RAGE, receptor for advanced glycation end product.
surface, as well as AGE-β,M-induced monocyte chemotaxis and tumor necrosis factor-α (TNF) expression. MP activation after exposure to AGE-β,M with production of TNF, was likely to result from oxidant-sensitive mechanisms, as shown by inhibition in the presence of N-acetylcysteine. Immunohistochemical studies confirmed staining for RAGE antigen in the mononuclear cells infiltrating amyloid deposits of long-term hemodialysis patients. Taken together, these data indicate a potential role of AGE-RAGE interaction in the pathogenesis of the inflammatory events associated with DRA.

Methods

Purification of nonglycated and AGE-β,M. Nonglycated and AGE-β,M were purified and characterized from the urine of long-term hemodialysis patients according to previously published methods (12, 13). Characteristic of AGES, AGE-β,M was brown in color and fluorescence (4, 29) and was further characterized as an AGE by its immunoreactivity with anti-AGE antibodies which recognize AGE-bovine serum albumin, AGE–human serum albumin, and AGE-hemoglobin, but not nonglycated products nor the early, reversible products of the Maillard reaction (12, 21).

Endotoxin removal from purified β,M and AGE-β,M. Endotoxin was removed from the in vivo isolated glycated and nonglycated forms of β,M as previously described (12). Endotoxin levels were measured by a kit from Seikagaku Corp. (Tokyo, Japan) and all materials used in these studies were subsequently found to be endotoxin free (<0.5 U/ml).

Purification of rat RAGE and preparation of antisera. Rat RAGE was purified to homogeneity from lung powder (Sigma Immunochemicals, St. Louis, MO) using methods similar to those described for the purification of bovine RAGE (14). Rat RAGE, which bears significant (>90%) homology to human RAGE, demonstrates immunoreactivity with anti-RAGE IgG and amino-terminal and internal sequencing indicated identity to the predicted sequence for rat RAGE based on that deduced from the rat cDNA (Schmidt, A.M., unpublished observation). Based on ∼32,000 M, and comparison with RAGE purified from bovine lung, rat lung RAGE also most likely represents the amino-terminal two-thirds of the molecule (i.e., the extracellular domain; 19) and is identified as soluble RAGE or sRAGE. Monospecific, polyclonal antisera to rat RAGE was purified by chromatography on protein A (Schleicher & Schuell, Keene, NH) according to the manufacturer’s instructions. F(ab')2 fragments were prepared from IgG using a kit from Pierce (Rockville, IL) according to the manufacturer’s instructions. Nonimmune IgG and F(ab')2 fragments were similarly prepared from sera derived from rabbits not immunized with RAGE.

Binding assays. AGE-β,M or nonglycated β,M was radiolabeled by the lactoperoxidase method (22) to a specific activity of 2.7 and 3.0 × 10⁸ cpm/ng, respectively, and bound to immobilized RAGE (5 µg/well, previously dissolved in bicarbonate/carbonate buffer [pH 9.6] after an overnight incubation at 4°C). Wells were subsequently washed with PBS (pH 7.4), blocked with PBS containing bovine serum albumin (1%), and then a binding assay was carried out with the indicated concentrations of radiolabeled protein with or without a 25-fold molar excess of the respective unlabeled protein for 3 h at 4°C. Where indicated, radiolabeled AGE-β,M was preincubated with the indicated concentration of soluble RAGE or human serum albumin, or MPs were preincubated with the indicated concentrations of anti-RAGE IgG or nonimmune IgG for 2 h at 4°C before addition of radiolabeled AGE-β,M. Elution was then performed with heparin-containing buffer as above.

Chromatography. Chemotaxis assays, performed in 48-well microchemotaxis chambers (Neuro-Probe, Bethesda, MD) containing polycarbonate membranes (Nucleopore, Pleasanton, CA), were performed as previously described (15) using human peripheral blood-derived MPs.

TNF-α assays. Human peripheral blood monocytes, isolated as above, were placed in the wells of 24-well tissue culture plates for 7 d in RPMI 1640 (Gibco Laboratories, Grand Island, NY) containing fetal bovine serum (10%; Gemini, Calabasas, CA). On day 8, wells were washed with PBS and incubated overnight in RPMI 1640 without serum. The following day, cells were incubated with indicated concentration of reagent for 16 h at 37°C, and supernatant was collected and assayed for TNF-α using an ELISA kit supplied by R&D Systems (Minneapolis, MN). Precipitated cells were treated with Triton (Gibco Laboratories), RNA was prepared, and RT-PCR analysis was performed as below. Where indicated, cells and/or wells were preincubated with various reagents as indicated.

RT-PCR analysis. Human primers for TNF-alpha and beta-actin were purchased from Stratagene (La Jolla, CA) and RT-PCR was performed according to the manufacturer’s recommendations.

Assay for reactive oxygen intermediates. The cytochrome c assay was performed using AGE-β,M or its nonglycated form according to previously described methods (16, 24).

Immunohistochemistry. Cutaneous tumor consisting of amyloid deposits was obtained postmortem from patients who had undergone hemodialysis for 16 yr. The sample was fixed in formalin (10%) and embedded in paraffin. After confirmation of the presence of amyloid material by Congo red staining and staining with anti-β,M antibody (13), sections were cut (2 µm) and mounted on slides and deparaffinized. Sections were then incubated with anti-RAGE IgG or nonimmune IgG according to previously published methods (14, 15) or incubated with anti-CD 68 IgG (Dako Corp., Carpinteria, CA) according to the manufacturer’s instructions. In other experiments, sections were stained with affinity-purified anti-AGE IgG as described (10).

Results

Binding of AGE-β,M to RAGE and MPs. 125I-AGE-β,M purified from the urine of patients with DRA bound to the extracellular domain of RAGE using the microtiter assay as described above. Specific, dose-dependent binding of radiolabeled AGE-β,M to immobilized RAGE was observed, with Kᵦ = 53.5 nM (Fig. 1 A), similar to that which was observed previously (14, 15, 19). Consistent with the specificity of 125I-AGE-β,M interaction with RAGE, preincubation of tracer with free sRAGE resulted in concentration-dependent inhibition of binding (Fig. 1 B). In contrast, equimolar concentrations of excess human serum albumin did not compete with binding of 125I-AGE-β,M to RAGE and radiolabeled nonglycated β,M displayed no specific binding to RAGE (data not shown). As AGE-β,M has been shown to induce monocyte activation, it was likely that it would bind to the cell surface in order to subsequently trigger cellular effector mechanisms. Incubation of 125I-AGE-β,M with MPs resulted in dose-dependent, specific binding, with Kᵦ = 81.6 nM (Fig. 1 C), similar to the affinity of binding observed previously with AGE albumin prepared in vitro (15). That RAGE was involved in the interaction of 125I-AGE-β,M and RAGE interaction and Amyloidosis 1089
AGE-β₂M with monocytes was supported by the inhibition of binding observed upon preincubation of tracer with increasing concentrations of sRAGE (Fig. 1D), but not in the presence of equimolar concentrations of human serum albumin (data not shown). Further support for the concept that RAGE is a central mediator of the interaction of AGE-β₂M with MPs was demonstrated in studies using anti-RAGE IgG. Anti-RAGE IgG, but not nonimmune IgG, largely inhibited the binding of 125I-AGE-β₂M to MPs in a dose-dependent manner (Fig. 1E).

*AGE-β₂M induced monocyte migration and TNF expression by MPs: role of RAGE.* We next considered whether the biologic sequelae of the interaction of AGE-β₂M with MPs were dependent on binding to RAGE. AGE-β₂M, but not its nonglycated form, mediated induction of monocyte chemotaxis when placed in the lower compartment of microchemotaxis chambers (Fig. 2, lines 3 and 2, respectively). Consistent with the hypothesis that this interaction was RAGE dependent, pretreatment of monocytes with anti-RAGE IgG or pretreatment of AGE-β₂M with sRAGE inhibited monocyte chemotaxis in a dose-dependent manner (Fig. 2, lines 6, 4, and 7, respectively). In contrast, nonimmune IgG (Fig. 2, line 5) or excess concentrations of albumin had no effect (data not shown).
A critical link in the association of RAGE with DRA would be the demonstration of enhanced expression of RAGE in the inflammatory cells in amyloid tissue from patients with DRA. In these studies, we investigated the expression of RAGE in amyloid tissue of patients with DRA using immunohistochemistry (Fig. 5 A) and antibodies to β2M (Fig. 5, B and F) confirmed that the massive deposits of amyloid tissue observed in a cutaneous tumor of a patient who had undergone hemodialysis for 16 yr were composed of β2M. Consistent with the concept that sites of amyloid were composed of reversibly glycated AGE-β2M, immunoreactivity with antibody to CD68 (Fig. 5, D and H), were positive for RAGE (Fig. 5, C and G). In contrast, immunoreactivity with nonimmune IgG was negative (data not shown).

Discussion

The interaction of AGEs with monocytes can potentially result from interaction with several cell surface polypeptides. These include p60 and p90 (25), identified in the homogenates of rat liver, the macrophage scavenger receptor (26), shown to bind glucose-modified extracellular membrane components,
and RAGE. The best-characterized of these is the latter, a member of the immunoglobulin superfamily expressed by cells important in the vasculature: MPs, endothelial cells, and smooth muscle cells, as well as by certain neurons and mesangial cells (27). RAGE has been shown to centrally mediate the interaction of AGE-modified proteins with endothelium, being responsible for the initial rapid phase of clearance observed after infusion of AGE-albumin and induction of IL-6 transcripts (18). This receptor also appears to have a central role in AGE-induced perturbation of endothelial properties, including diminished barrier properties after exposure to AGEs (28) and induction of vascular cell adhesion molecule-1 expression (17). In the context of the biology of MPs, RAGE has a central role in binding of AGEs, and subsequent changes in cell migration (15).

Although it might be expected that RAGE expression is linked exclusively to hyperglycemic states, situations in which AGEs are abundant, tissue surveys have indicated a more complex picture. While RAGE and AGEs are upregulated in diabetic vasculature (29), RAGE is also expressed at high levels in developing brain and appears to serve as a receptor for the neurite-promoting polypeptide amphoterin, the latter being coexpressed in neonatal nervous tissue (30).

Another situation in which RAGE expression is enhanced accompanies immune/inflammatory disorders (Schmidt, A.M., unpublished observations). The data presented here suggest that an irreversibly glycated form of the circulating plasma protein β2-M, the clearance of which is markedly delayed in the setting of renal failure and hemodialysis which accumulates in the tissues and joints of certain individuals, interacts with RAGE on the surface of MPs. In addition to specific, largely RAGE-dependent binding, AGE-β2-M-RAGE interaction mediates monocyte chemotaxis as well as the induction of cytokines such as TNF-α by these cells. Although one might posit that this result is expected based on observations concerning RAGE and other glycated proteins, this is the first study examining cellular interactions of AGEs prepared from nondiabetic tissues. As AGEs are an heterogeneous class of structures which form under quite distinct conditions, it is important to determine in each situation the impact of nonenzymatic glycation on properties of the affected macromolecules, as well as their ability to bind to cellular surfaces.

The implications of AGE-β2-M binding to monocyte RAGE may be relevant to the pathogenesis of DRA. We have observed upregulation of RAGE in MPs of amyloid tissue of long-term hemodialysis patients and postulate that this critical inflammatory event in amyloid deposits initiates a cascade of events leading to the induction of matrix metalloproteases (31, 32), as well as other cytokines such as interleukin-6 (33, 34), ultimately priming the affected bones and joints for the development of periarticular soft tissue swelling, diffuse destructive arthropathy, and subchondral bone erosions and cysts characteristic of this clinical syndrome (35–37).

One property common to AGEs is the generation of reactive oxygen intermediates, which, after their tethering to the cell surface, produces oxidant stress as a means of perturbing target cells; for example, oxidant stress underlies activation of NF-kB and diminished barrier function consequent to binding of AGEs to endothelial RAGE (16, 17, 28). AGE-β2-M interaction with RAGE on monocytes is likely to activate cells by similar mechanisms, as N-acetylcysteine blocked AGE-induced monocyte expression of TNF, and AGE-β2-M, by itself, reduced cytochrome c. The central role of the AGEs in the generation of enhanced oxidant stress is suggested by studies in which incubation of amphoterin, a nonglycated ligand of RAGE (30), with endothelial cells or cultured neurons does not result in increased production of thiobarbituric acid reactive substances upon ligation of cellular RAGE (data not shown). Consistent with this concept, pilot studies have demonstrated that incubation of amphoterin with MPs does not result in increased production of TNF (data not shown).

These studies represent the first indication that RAGE may play a role in the development and perpetuation of certain inflammatory events, such as DRA. In addition, as recent data have revealed enhanced RAGE expression in the affected vasculature of individuals with inflammatory vasculitides, a circumstance in which AGEs are less likely to form, this strongly suggests that RAGE may interact with other ligands in certain pathological settings. In this context, we have identified a 12-kD polypeptide which binds RAGE and appears to be upregulated at sites of inflammation (Schmidt, A.M., unpublished observations). As with other immunoglobulin superfamily receptors, such as intercellular adherence molecule-1 which also has multiple ligands (38), elucidation of the biology of RAGE in varied pathologic settings will require identifica-

Figure 4. AGE-β2-M mediates the generation of oxidant stress: effect of N-acetylcysteine. AGE-β2-M, but not its nonglycated form (50 μg/ml), results in reduction of cytochrome c (A) as described above, a process partially inhibited in the presence of superoxide dismutase (data not shown). Data shown represent the mean±SEM of triplicate determinations. Experiments were repeated twice. (B) MPs, isolated and prepared as above, were incubated with the indicated concentrations of N-acetylcysteine, and supernatants were harvested and assayed for TNF. Results are expressed as percent maximal induction of TNF (279.45±29.67 pg/ml) as mean±SEM of duplicate determinations.
tion of the biologically relevant ligand, and dissection of the consequences of receptor activation. Intense investigation is ongoing to characterize the nature of AGE and non-AGE ligands of RAGE, as understanding these interactions is essential to define the factors which impact on and regulate RAGE expression. The results of these studies represent a critical step in the design of therapies (such as attenuating AGE formation, blocking AGE-RAGE interaction, and/or diminishing AGE-RAGE–mediated enhanced cellular oxidant stress) intended to mitigate the diverse effects of RAGE in different pathologic settings.

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