Advanced Glycation End Products Activate Endothelium Through Signal-Transduction Receptor RAGE

A Mechanism for Amplification of Inflammatory Responses

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Background—The products of nonenzymatic glycation and oxidation of proteins, the advanced glycation end products (AGEs), form under diverse circumstances such as aging, diabetes, and kidney failure. Recent studies suggested that AGEs may form in inflamed foci, driven by oxidation or the myeloperoxidase pathway. A principal means by which AGEs alter cellular properties is through interaction with their signal-transduction receptor RAGE. We tested the hypothesis that interaction of AGEs with RAGE on endothelial cells enhances vascular activation.

Methods and Results—AGEs, RAGE, vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and E-selectin are expressed in an overlapping manner in human inflamed rheumatoid synovia, especially within the endothelium. In primary cultures of human saphenous vein endothelial cells, engagement of RAGE by heterogeneous AGEs or \(N^\epsilon\)-(carboxymethyl)lysine–modified adducts enhanced levels of mRNA and antigen for vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and E-selectin. AGEs increased adhesion of polymorphonuclear leukocytes to stimulated endothelial cells in a manner reduced on blockade of RAGE.

Conclusions—AGEs, through RAGE, may prime proinflammatory mechanisms in endothelial cells, thereby amplifying proinflammatory mechanisms in atherogenesis and chronic inflammatory disorders. (Circulation. 2002;105:816-822.)

Key Words: cell adhesion molecules ■ receptors ■ endothelium ■ inflammation ■ vasculature

The products of nonenzymatic glycation and oxidation of proteins, the advanced glycation end products (AGEs), are a heterogeneous class of compounds that form under diverse circumstances.\(^1\)–\(^8\) AGEs, and, specifically, \(N^\epsilon\)-(carboxymethyl)lysine (CML) adducts of proteins, the most prevalent AGE detected in vivo,\(^9\)–\(^11\) may form in milieu characterized by oxidative stress. Specifically, AGE- and CML-modified adducts have been identified in atherosclerotic lesions of rabbits; their expression colocalized with that of oxidation epitopes, such as malondialdehyde and 4-hydroxynonenal.\(^12\)\(^,\)\(^13\) Furthermore, the observation that activation of the myeloperoxidase–hydrogen peroxide–chloride system leads to conversion of hydroxy-amino acids into glycolaldehyde, a precursor in the steps leading to formation of CML,\(^14\) suggested that AGEs may form in inflammatory milieu.

The receptor for AGE (RAGE), a member of the immunoglobulin superfamily of cell surface molecules, is a central signal-transduction receptor for AGEs such as CML-modified adducts. Engagement of RAGE by these ligands activates key signal transduction pathways, such as p21\(^\text{ras}\), erk 1/2 kinases and nuclear factor-\(\kappa\)B in endothelial cells, monocytes, and vascular smooth muscle cells. This cascade of events leads to RAGE-mediated–enhanced expression of proinflammatory mediators as demonstrated by suppression of the effects of these modified adducts in the presence of blocking antibodies to RAGE, soluble RAGE, the extracellular ligand-binding domain of the receptor, or by transient transfection of cDNA encoding cytosolic tail-deleted RAGE into RAGE-bearing cells.\(^15\)\(^–\)\(^17\) These studies provided critical evidence that both ligand engagement and signal transduction through RAGE were importantly involved in mediating the effects of AGEs.\(^17\)

Multiple reports have suggested that sustained accumulation of RAGE ligands leads to enhanced expression of the receptor; one mechanism underlying this observation probably is the presence of at least two functional binding elements for nuclear factor-\(\kappa\)B in the promoter of the gene encoding RAGE.\(^18\)\(^–\)\(^20\) For example, in vasculature retrieved from subjects with diabetes and in joint tissue from subjects with

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kidney failure and dialysis-related amyloidosis, increased accumulation of AGE epitopes and their colocalization with increased RAGE antigen was evident in the blood vessels and synovia, respectively, compared with age-matched control subjects.21,22 These observations, together with those linking AGE formation to neutrophil activation in the myeloperoxidase–hydrogen peroxide–chloride system, prompted us to test the concept that AGEs were present in inflamed foci in the absence of diabetes and kidney failure and that interaction of AGEs with RAGE activated endothelial cells, thereby providing a mechanism for amplification of inflammatory responses in diverse milieux.

Methods

Endothelial Cell Culture

Human saphenous vein endothelial cells (HSVECs) were harvested and characterized as described.23 Cells were maintained in Medium 199 (Life Sciences) containing fetal bovine serum (5%) and growth factors (50 U/mL heparin and 50 μg/mL endothelial cell growth factor). Cell number was determined by direct cell counting of adherent cells after detachment by trypsin with the use of a hemocytometer, and viability was assessed by exclusion of trypan blue.

Preparation of AGEs and CML Adducts of Proteins

Bovine serum albumin, ovalbumin, and lipopolysaccharide (LPS) derived from Escherichia coli were obtained from Sigma. Bovine serum albumin (25 mg/mL) was incubated in the presence of d-ribose (25 mg/mL) under sterile conditions at 37°C for 6 weeks in phosphate buffer (0.2 mol/L), pH 7.4. AGEs were then dialyzed extensively against PBS.15,24 Nonglycosylated bovine serum albumin was subjected to the same conditions except that d-ribose was omitted. Reagents were characterized as AGEs by ELISA with the use of affinity-purified anti-AGE IgG.24 To obtain AGEs from human subjects, serum was obtained from patients with uremia. AGEs were immunosolated by chromatography of serum onto Affi-gel 10 resin (Bio-Rad) to which affinity-purified anti-AGE IgG had been immobilized. The material that did not adhere to the column did not display immunoreactivity for AGEs by ELISA (with the use of affinity-purified anti-AGE IgG). The material eluted in the presence of NaCl (2 mol/L) was dialyzed versus PBS and demonstrated immunoreactivity for AGEs by ELISA.24 To inhibit the effects of AGEs, monospecific rabbit anti-human RAGE IgG was used.15 CML-modified ovalbumin (90 nmol CML/mmol lysine) was prepared and characterized as described.17 AGEs and CML-modified adducts contained undetectable levels of LPS (<3 pg/mL) by Limulus amebocyte assay (Sigma).

Detection of Cell Surface Adhesion Molecules

Assay of cell surface molecules was performed by cell surface immunocytochemistry. The following mouse anti-human monoclonal antibodies were used: anti–vascular cell adhesion molecule (VCAM)-1 IgG (Ab E1/6), anti–E selectin IgG (Ab H18/7), anti–intercellular adhesion molecule (ICAM)-1 IgG (Ab HU5/3), major histocompatibility complex class I (MHC-I W6/32), and the monoclonal antibody E1/1, recognizing a constitutive and non–cytokine-inducible endothelial cell antigen.20 Enzyme immunocytoassays were carried out by incubating the endothelial cell monolayers with saturating concentrations of specific monoclonal antibodies against the target molecule (undiluted supernatants from the hybridoma cells), followed by biotinylated goat anti-mouse IgG, and streptavidin–alkaline phosphatase (Amersham Life Sciences). The surface expression of each adhesion molecule was quantified spectrophotometrically (optical density at 405 nm wavelength).

Cell Adhesion Assays

Monocytoid U937 cells were obtained from American Type Tissue Culture Collection (Rockville, Md). Human polymorphonuclear leukocytes were isolated from blood of healthy volunteers through the use of Dextran T-500 (Pharmacia) and Ficoll-Hypaque (Lymphoprep, Nycomed). HSVEC were grown to confluence and AGEs were added for 6 hours. Adhesion assays were performed as described.27,28

Northern Blotting

Total cellular RNA was isolated by single extraction through the use of the acid guanidinium thiocyanate–phenol–chloroform method.29 Electrophoresis of isolated RNA was performed with 30 μg RNA/ lane, separated on a 1% agarose-formaldehyde gel. Contents of the gels were transferred to nylon membranes (Amersham Hybond-N) and immobilized by short-wave UV illumination. Membranes were prehybridized for 2 hours before hybridization with 32P-labeled DNA probes labeled by random hexanucleotide priming (Pharmacia) to specific activities >108 cpm/μg DNA and autoradiographed; 18S and 28S ribosomal RNA fluorescence intensity of the ethidium–bromide–stained membranes served as control for RNA loading. Quantification of densities of autoradiographic bands for Northern hybridization was performed with the NIH Image 1.6 software.

Immunohistochemistry

Synovial tissue was retrieved from two subjects with rheumatoid arthritis. Tissues were fixed in formalin (10%) and processed with paraffin embedding. Sections (4 μm thick) were incubated with trypsin for 8 minutes at 37°C and then processed by a Dako EnVision Peroxidase mouse kit with a biotin streptavidin–amplified detection system after preliminary incubation with the specific polyclonal or monoclonal antibodies. In addition to the above-described antibodies, anti-L26 (PanB), anti–UCHL1 (pan T), anti–CD68, and anti–thymoglobin (Dako) were used.

Statistical Analysis

Multiple comparisons were performed by ANOVA and individual differences tested by the Fisher’s protected least significant difference test after the demonstration of intergroup differences by ANOVA. Two-group comparisons were performed by means of the unpaired Student’s t test. Comparisons of distribution of fluorescent intensities at flow cytometry were performed by the Kolgomorov–Smirnov statistic with the aid of a statistics software package from Becton Dickinson.

Results

To test the concept that AGEs, and particularly CML-modified adducts, may form and interact with RAGE in an inflammatory milieu, we first tested the premise that AGEs accumulated in inflamed tissue. In inflamed synovial tissue from two subjects with rheumatoid arthritis, immunohistochemistry revealed accumulation of AGE epitopes in the blood vessel wall and surrounding connective tissue (Figures 1 and 2, a). RAGE antigen was expressed in cells of the blood vessel wall, particularly within the endothelium (Figures 1 and 2, b), in a manner overlapping with the distribution of AGE epitopes.

Although other cell types within rheumatoid synovia express AGES and RAGE, such as vascular smooth muscle and cells of the interstitium, we focused on the potential proinflammatory implications of endothelial expression of these molecules.17,30 VCAM-1, ICAM-1, and E-selectin were expressed in the endothelia in human rheumatoid synovia (Figures 1 and 2, c, d, and e, respectively) and colocalized with AGE epitopes and RAGE antigen. Control experiments with anti–thymoglobin IgG (Figures 1 and 2, f) and anti–L26
pan B), anti-UCHL1 (pan T), and anti-CD68 IgG (data not shown) failed to demonstrate the presence of these antigens in the endothelium of rheumatoid synoviae (Figures 1 and 2, f). These observations in two distinct human subjects with rheumatoid arthritis supported the hypothesis that AGEs and RAGE were present in the vascular endothelium at sites of inflamed foci.

To test the concept that AGEs may upregulate expression of proinflammatory adhesion molecules, we studied expression of these molecules in AGE-stimulated endothelial cells. We used primary cultures of HSVEC. Northern blotting revealed that compared with HSVEC incubated with native albumin (Figure 3, a, b, and c; lane 1), HSVEC treated with AGE-albumin (600 μg/mL) for 4 hours at 37°C in the presence/absence of pretreatment of the monolayer with the indicated IgG (70 μg/mL) for 2 hours at 37°C. Cells were harvested, RNA was prepared, and Northern blotting was performed. To control for relative amounts of RNA loading, densitometric analysis was performed with ethidium bromide–identified 28S ribosomal RNA bands. In these experiments, “1.0” is arbitrarily assigned to bands from cells treated with native albumin. Three Northern blots per condition were performed with analogous results; representative blots are shown. Mean±SD is shown.

Figure 1. AGEs, RAGE, VCAM-1, ICAM-1, and E-selectin are expressed in the vasculature of rheumatoid synovia. Synovial tissue was retrieved from two nondiabetic human subjects with rheumatoid arthritis. Tissue was fixed and immunostaining performed with affinity-purified anti–AGE IgG (8 μg/mL) (a); anti–human RAGE IgG (60 μg/mL) (b); or undiluted supernatant from hybridoma cells expressing anti–VCAM-1 IgG (c); anti–ICAM-1 IgG (d); anti–E-selectin IgG (e); and anti–thyroglobulin IgG (f) (1:500 dilution of hybridoma supernatant). Immunohistochemistry with the indicated nonimmune IgG revealed no specific staining (not shown). Scale bar=8 μm.

Figure 2. AGEs, RAGE, VCAM-1, ICAM-1, and E-selectin are expressed in the vasculature of rheumatoid synovia. Synovial tissue was retrieved from two nondiabetic human subjects with rheumatoid arthritis. Tissue was fixed and immunostaining performed with affinity-purified anti–AGE IgG (8 μg/mL) (a); anti–human RAGE IgG (60 μg/mL) (b); or undiluted supernatant from hybridoma cells expressing anti–VCAM-1 IgG (c); anti–ICAM-1 IgG (d); anti–E-selectin IgG (e); and anti–thyroglobulin IgG (f) (1:500 dilution of hybridoma supernatant). Immunohistochemistry with the indicated nonimmune IgG revealed no specific staining (not shown). Scale bar=18 μm.

Figure 3. Incubation of HSVEC with AGEs enhances steady-state levels of mRNA for VCAM-1, ICAM-1, and E-selectin. HSVEC were incubated with native albumin or AGE-albumin (600 μg/mL) for 4 hours at 37°C in the presence/absence of pretreatment of the monolayer with the indicated IgG (70 μg/mL) for 2 hours at 37°C. Cells were harvested, RNA was prepared, and Northern blotting was performed. To control for relative amounts of RNA loading, densitometric analysis was performed with ethidium bromide–identified 28S ribosomal RNA bands. In these experiments, “1.0” is arbitrarily assigned to bands from cells treated with native albumin. Three Northern blots per condition were performed with analogous results; representative blots are shown. Mean±SD is shown.
Incubation of HSVEC with AGEs results in enhanced expression of cell surface VCAM-1, ICAM-1, and E-selectin. HSVEC were incubated with the indicated concentrations of native albumin, AGE albumin, or in vivo-derived uremic AGEs. For 16 hours for detection of VCAM-1 and ICAM-1 or 6 hours for detection of E-selectin at 37°C in the presence/absence of pretreatment (2 hours) with either anti-RAGE or nonimmune IgG (70 μg/mL). At the end of the incubation period, cells were incubated with undiluted hybridoma supernatants containing anti-VCAM-1 IgG (a), anti-ICAM-1 IgG (b), or anti-E-selectin IgG (c). Sites of primary antibody binding were identified by alkaline phosphatase–conjugated anti-murine IgG. Mean ± SD of optical density units at 405 nm of at least 8 replicates is reported.

Importantly, although incubation of HSVEC with LPS markedly increased expression of VCAM-1, ICAM-1, and E-selectin (Figure 4b, a, b, and c, respectively; lane 13), these effects were not due to ligation of RAGE. Pretreatment of the LPS monolayers with anti–RAGE IgG had no effect on expression of these adhesion molecules (Figure 4, a, b, and c; lane 14). These findings underscored the concept that RAGE was not a cell surface receptor for broad classes of proinflammatory mediators in the vessel wall but a specific receptor for its ligands, such as AGE-/CML-modified adducts.

Because AGEs represent a heterogeneous group of structures, it was essential to determine if CML-modified adducts of proteins might exert similar effects on expression of adhesion molecules. Incubation of HSVEC with CML-ovalbumin resulted in dose-dependent increases in cell surface expression of VCAM-1, ICAM-1, and E-selectin (Figure 5, a, b, and c, respectively; lanes 2–4) compared with native albumin (Figure 5, a, b, and c, respectively; lane 1). That these effects of CML-ovalbumin were mediated largely by RAGE was demonstrated by their ability to induce increased expression of cell surface adhesion molecules. Compared with incubation of HSVEC with native albumin, exposure of HSVEC to AGE-albumin resulted in dose-dependent increases in cell surface expression of VCAM-1 (Figure 4a, lanes 1–2 to 3–4, respectively). That these effects were mediated in part by RAGE was suggested by reduction in AGE-mediated increased levels of VCAM-1 by anti–RAGE IgG but not nonimmune IgG (Figure 4a, lanes 7 and 8, respectively). These proinflammatory effects of AGEs were completely suppressed on heat inactivation of AGE-modified albumin (data not shown). Incubation of HSVEC with in vivo–derived AGEs, purified from the serum of patients with kidney failure, resulted in an ∼4-fold increase in cell surface expression of VCAM-1 (Figure 4a, lane 10) compared with native albumin (Figure 4a, lane 9). Although nonimmune IgG had no effect on uremic AGE-mediated increases in VCAM-1 expression, incubation of HSVEC with anti–RAGE IgG significantly decreased expression of cell surface VCAM-1 (Figure 4a, lanes 12 and 11, respectively).

To assess regulation of cell surface expression of ICAM-1 by the interaction of AGEs with RAGE, HSVEC were incubated with AGE albumin. A dose-dependent increase in cell surface expression of ICAM-1 was noted compared with native albumin (Figure 4b, lanes 2, 3, 4, and 1, respectively). This effect was mediated at least in part by RAGE, as demonstrated by decreased AGE-mediated expression of ICAM-1 in the presence of anti–RAGE IgG but not by nonimmune IgG (Figure 4b, lanes 7 and 8, respectively). In vivo–derived uremic AGEs had similar effects; compared with native albumin, incubation of HSVECs with uremic AGEs resulted in an ∼1.7-fold increase in cell surface expression of ICAM-1 (Figure 4b, lanes 9 and 10, respectively). These effects were mediated in part by RAGE, as demonstrated by reduced expression of ICAM-1 in the presence of anti–RAGE IgG but not by nonimmune IgG (Figure 4b, lanes 11 and 12, respectively). Because E-selectin mediates targeting of inflammatory cells, particularly polymorphonuclear leukocytes (PMN), to activated endothelia, we tested the role of AGEs in upregulating expression of this adhesion molecule. Compared with native albumin, incubation of endothelial cells with AGE albumin or in vivo–derived uremic AGEs resulted in increased cell surface expression of E-selectin (Figure 4c, lanes 1 and 9 and 2–4 and 10, respectively). These effects of AGEs were mediated in part by RAGE, as demonstrated by decreased expression of E-selectin in the presence of anti–RAGE IgG (Figure 4c, lanes 7 and 11) but not by nonimmune IgG (Figure 4c, lanes 8 and 12).
significantly decreased cell surface expression of VCAM-1, ICAM-1, and E-selectin in the presence of anti–RAGE IgG but not nonimmune IgG (Figure 5, a, b, and c, respectively; lanes 6 and 7, respectively).

Previous studies demonstrated that incubation of AGEs with endothelial cells resulted in enhanced adhesion of mononuclear cells to activated endothelia.17,30 The key issue in the present studies was to determine whether engagement of RAGE by AGEs on HSVEC would increase adhesion of PMNs. Compared with incubation of HSVEC with native albumin, incubation of the monolayers with AGE albumin resulted in an ~5-fold increase in numbers of adherent PMNs to HSVEC (Figure 6, a and b, respectively; see comparisons in Figure 6g). This effect was dependent, at least in part on RAGE, as demonstrated by significantly reduced numbers of adherent PMN in the presence of anti–RAGE IgG (Figure 6, c and g) but not nonimmune IgG. Consistent with earlier studies,30 incubation of HSVEC with AGEs resulted in increased adhesion of mononuclear U937 cells, in a manner reduced by anti–RAGE IgG (data not shown).

**Discussion**

Recent studies have suggested that the biology of AGEs extends beyond settings such as diabetes, kidney failure, and atherosclerosis. The observation that AGEs such as CML form in inflammatory lesions, such as in the joints of patients with dialysis-related amyloidosis,6,7 or in highly-oxidized states, such as in atherosclerotic lesions,12,13 suggested that the potential biological effects of these modified adducts may be far-reaching. Formation of CML adducts may be driven by the myeloperoxidase pathway, indicating that release of activated neutrophils in inflammatory milieu may stimulate further generation of CML-modified proteins and lipids.14 Indeed, the observation that active myeloperoxidase is present in human atherosclerotic lesions31 supports the view that in locally inflamed foci, sustained generation of AGEs might provide a stimulus for ongoing cellular perturbation.
In this context, distinct proinflammatory ligands of RAGE, S100/calgranulins, are present in inflammatory cells such as PMNs, monocytes, and lymphocytes. Once attracted to sites of inflammation, S100/calgranulins may be released from these cells, thereby allowing them to engage RAGE. Once in the extracellular space with access to cell surface receptor, these molecules might amplify inflammatory responses through enhanced generation of proinflammatory adhesion molecules, cytokines, and tissue-destructive matrix metalloproteinases. We speculate that in the inflammatory cascade amplified by RAGE, attraction of PMNs to sites of inflammation, at least in part due to release of highly proinflammatory S100/calgranulins engaging the receptor, primes processes leading to ongoing generation of AGEs, and, in particular, CML-modified structures. Continued generation of AGE and CML-modified adducts may lead to sustained expression of proinflammatory mediators on the endothelial surface, leading to a vicious cycle of attraction and activation of inflammatory effector cells to inflamed foci, orchestrated, at least in part, by RAGE.

In contrast to cellular activation mediated by LPS, where marked, but often short-lived upregulation of inflammatory effector molecules occurs, engagement of RAGE by S100/calgranulins and AGEs/CML results in a smoldering degree of chronic cellular stimulation. In the long term, we speculate that engagement of RAGE by these ligands may underlie continued amplification of inflammatory events in tissues previously sensitized by lipid deposition or by immune/inflammatory triggers. If physiological mechanisms of tissue repair fail to arrest these proinflammatory signals, if left unchecked, such sustained cellular activation mediated by RAGE might eventuate in tissue injury. These findings extend the proinflammatory paradigm involving RAGE by suggesting that ongoing generation of AGEs in inflamed foci might amplify cellular perturbation and tissue damage by engagement of this receptor on the endothelial surface.

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