Advanced Glycation End Products Modulate the Maturation and Function of Peripheral Blood Dendritic Cells

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Advanced glycation end products (AGEs), a complex and heterogeneous group of posttranslational modifications of proteins in vivo, have been widely studied for their involvement in diabetic complications; these complications are largely vascular and accompanied by inflammation. Because dendritic cells (DCs) initiate and modulate inflammatory responses, we hypothesized that AGEs might exert immunomodulatory effects via antigen-presenting DCs. To test this hypothesis, we investigated effects of the AGE peptide, compared with the naked peptide, on maturation, costimulatory molecule expression, and function of DCs in peripheral blood. From flow cytometry, we found a dose-dependent inhibition in CD83 expression on DCs exposed for 2.5 h to each of two synthetic AGE peptides. A similar culture for 24 h additionally produced an inhibition of CD80 expression, whereas exposure to AGEs for 3 days induced a large increase in DC numbers and a concomitant loss of monocyte/macrophages. Exposure of DCs to AGEs resulted in a dose-dependent loss in their capacity to stimulate primary proliferation of allogeneic T-cells. We conclude that AGEs promote development of DCs but that these DCs fail to express maturation markers and lose the capacity to stimulate primary T-cell responses. Effects of AGEs on DCs could be instrumental in the immunological changes associated with diabetes. Diabetes 53:1452–1458, 2004

Advanced glycation end products (AGEs) result from irreversible changes to long-lived proteins in the body. Reducing sugars and their metabolites react with free amino groups (the Maillard reaction) and sequentially alter to produce complex heterogeneous adducts termed AGEs. Carbonyl compounds from glycolytic intermediates of Maillard reactions (1), including glyoxal and methylglyoxal (MG), are major contributors to AGE formation. MG is the most reactive AGE precursor, and its concentration is increased in diabetic subjects (2). MG reacts irreversibly with lysine to form glycosylamine protein cross-links and with arginine to give imidazolone derivatives (3).

AGEs are associated with complications of rheumatoid arthritis, Alzheimer's disease, and diabetes. Hyperglycemia in diabetes increases rate of formation of AGEs (4). Build up of adducts, and associated cross-links, on long-lived proteins such as collagen, crystallins, and vascular basement membranes may contribute to the complications of diabetes, including retinopathy, vascular hypertension, and nephropathy (5). AGEs are also measurable in blood in association with circulating proteins (6). Levels are low in healthy subjects but higher in subjects with diabetes. Elimination of AGE-modified protein is by catabolism and subsequent renal excretion, with levels being elevated in renal failure (7). Therefore, there is opportunity for AGE-modified proteins to come in contact with components of the immune system.

Dendritic cells (DCs) are antigen-presenting cells of the immune system, unique in their potency at stimulating B- and T-cells in primary immune responses (8). DCs and their precursors are present in blood. Blood and tissue DCs are regarded as being “immature.” Tissue DCs acquire and process antigens, mature, and migrate to lymph nodes, where they can stimulate lymphocytes (9). Markers expressed by DCs include the antigen presentation molecules major histocompatibility class I, class II, and CD1; adhesion molecules such as CD54; and the costimulatory molecules CD80 and CD86. Another marker traditionally thought of as a marker of DC maturation is CD83, a 45-kDa type I membrane glycoprotein. CD83 is not seen on resting blood leukocytes at detectable levels but may be rapidly upregulated. However, it is not limited to DCs. Recent reports show expression in a range of cells including polymorphonuclear neutrophils (10) and B-cells (11). Neither is CD83 just a marker of activation; it is a sialic acid–binding lectin adhesion receptor that binds monocytes and a subset of activated CD8+ T-cells (12). Preventing surface CD83 expression inhibits DC-mediated T-cell stimulation (13) and CD4+ T-cell development in the thymus (14).

The best characterized AGE receptor is the receptor for AGE (RAGE). RAGE-ligand interaction results in a signaling cascade to release proinflammatory cytokines, growth factors, and tissue factors (15).

Glycation of albumin in vivo is consistent with only one
to three glucose residues per protein molecule, making it appropriate to study such minimally glycated models in vitro (2). Proteins can be minimally glycated with MG to produce methylglyoxal-derived hydroimidazolone (MG-H) (16,17). The aim of this study was to use this adduct as a model to test for immunomodulatory effects of AGEs. The complications of diabetes are largely vascular, and vascular disease is accompanied by leukocyte activation and inflammation (18). Because DCs initiate and modulate inflammatory responses, we chose to investigate the interaction between AGEs and these primary antigen-presenting cells. In patients with diabetes, there is both an increase in DC numbers and an impaired ability of DCs to stimulate primary immune responses (19–21). We studied both of these aspects and report that AGEs modify the numbers, phenotype, and function of DCs.

**RESEARCH DESIGN AND METHODS**

**Preparation of peptides with minimal content of glycation adducts.** The peptides adrenocorticotropic hormone (ACTH) fragment 4–10, angiotensin I (AI), and MG (40% aqueous solution) were purchased from Sigma (Poole, U.K.).

Peptide (2.4 mmol/l) was dissolved in sodium phosphate buffer (1 ml, 0.02 mol/l, pH 7.8) containing MG (500 mmol/l) under aseptic conditions. The reaction mixture (RM) was incubated at 37°C for 24 h in a 2-ml glass vial and stored in the reaction vessel at 4°C. An endotoxin test revealed insignificant contamination in these reagents (Charles River Endosafe LAL test kit; Wilmington, MA).

**Mass spectrometric analysis of RMs.** The RMs (2 μl) were diluted 1:5 with distilled water, further diluted with 0.1% trifluoroacetic acid in water, mixed with freshly prepared α-matrix (α-cyan-o-hydroxycinnamic acid, 100 mg/ml in 0.1% trifluoroacetic acid in water/acetonitrile/methanol, 1:1:1), and applied to the target plate. Samples were analyzed on the Micromass ToFSpec 2E MALDI-MS.

**Isolation of peripheral blood mononuclear cells.** Heparinized human peripheral blood was obtained after informed consent was granted, diluted with medium (Dutch modified RPMI 1640 containing 100 units/ml penicillin/streptomycin and 2 mmol/l-glutamine [Sigma]), layered onto an equal volume of Ficoll Paque (Amersham Biosciences, Chalfont St. Giles, U.K.), and centrifuged (629g, 30 min, 20°C). Mononuclear cells were aspirated from the interfaces, washed twice (medium containing 10% FCS [Sigma]), spun at 600g (min), counted (trypan blue exclusion), and suspended at 3.5 million/ml.

For binding studies using MALDI-MS, and as stimulator cell populations for mixed leukocyte reactions, DCs were enriched from nonadherent mononuclear cell populations, after overnight incubation, by centrifuging at 600g over a 1.45% metrizamide gradient (Sigma).

**Exposure of cells.** Freshly isolated peripheral blood mononuclear cells (PBMCs) were exposed to sodium phosphate buffer (5 mmol/l AGE RM) or AGE, 250–500 μmol/l AGE, 250–500 μmol/l each); placed in a sterile six-well plate; and incubated at 37°C/5% CO2 in air. At times of 4, 24, and 48, and 69 h after addition of controls or AGE, 250-μmol aliquots were analyzed using flow cytometry. The same protocol was followed for culture with ACTH (fragment 4–10) and ACTH-AGE. Addition of 50-μl control beads to each sample before acquisition allowed calculation of relative cell numbers.

**RESULTS**

**Exposure of PBMCs to ACTH (fragment 4–10)-AGE RM.** ACTH (fragment 4–10) RM analyzed by mass spectrometry was found to contain the starting peptide and a number of adducts at higher masses (Fig. 1A). PBMCs were incubated with sodium phosphate buffer or ACTH (fragment 4–10) solution as controls or with one of two concentrations of ACTH (fragment 4–10)-AGE RM.

DCs were defined as DR+/CD3 and CD19)/CD14+ cells and monocytes as DR+/CD3 and CD19)/CD14+, excluding cells of other lineages (Fig. 2). Expression of other markers was then assessed (e.g., CD3, Fig. 2). Phenotypes of DCs exposed to either control were similar. Samples incubated with either concentration of AGE RM showed a similar percentage expression of CD11c, CD40, and CD86 (data not shown) to that of the control cells, but a complete lack of significant CD83 expression (Fig. 3).

Similar trends of surface marker expression were seen in monocyte populations. Cell aggregation and some cell death were seen in samples incubated with ACTH-AGE RM, but not in controls.

**Exposure of PBMCs to AI-AGE RM.** The desired MG-H adduct was seen in the AI-AGE RM as appropriate—54 μm higher than starting peptide (Fig. 1B). Within PBMCs exposed to AI-AGE RM or controls, CD11c, CD40, and CD86 expression on DCs was analogous. Surface CD83 was comparable on control samples, poorly expressed on 2-μl/ml AGE RM–exposed cells, and absent from the 5-μl/ml sample (Fig. 4A). Similar trends were again seen in CD14+ monocyte populations. AI-AGE–exposed cells showed only slight aggregation, and gated DCs and monocytes by flow cytometry showed little evidence for cell loss at the 2.5-h time point.
Expression of CD83 on AGE-exposed cells is dose dependent. Among the DC population, expression of surface CD83 was inversely proportional to concentration of AGE RM added to culture, within standard errors (Fig. 5A). Monocyte expression of CD83 did not drop until a higher concentration of AGEs had been added, and the decrease was less gradual than for those in the DC region (data not shown).

AGEs prevent upregulation of CD83 on preincubated DCs. PBMCs were isolated and incubated at 37°C/5% CO₂ in air for 2 h so that they would express a reference level of CD83 (just over 30% positive). These cells were divided into four equal parts, to which buffer, AI, or one of the two concentrations of AI-AGE RM was added. Cells were incubated for a further 2 h. DCs within control samples showed an increase in percentage of CD83 expression from the reference level, whereas those incubated with AGEs showed no such upregulation (Fig. 5B). In the CD14⁺ population, controls again showed a rise from the reference level, whereas AGE-exposed cells showed a reduction, falling to 65% from 80% expression in the reference sample.

Specificity of effect of AGEs. PBMCs were isolated and exposed to controls or AGEs and to controls or AGEs with added lipopolysaccharide (1 μg/ml) to upregulate surface markers. Cultures were left for 2, 4, or 24 h at 37°C/5% CO₂ in air. Cells were stained with monoclonal antibodies to CD40, CD80, CD45, and HLA-DR alongside the usual cocktail for population discrimination.

Neither DCs nor monocytes showed a significant difference in percentage expression of any markers after AGE exposure, except for lower CD80 expression (~50% less) after 24 h compared with controls. CD80 was not expressed significantly after 2 or 4 h.

AGE-exposed DCs have reduced capacity to stimulate a mixed leukocyte reaction. Cells treated with AI or buffer equally stimulated allogeneic lymphocytes (Fig. 6). LDCs exposed to 2 μl/ml AGE RM produced a low level of allogeneic stimulation, but proliferation was significantly higher than that in responders alone \( (P = 0.05) \), whereas those incubated with 5 μl/ml AGE showed no stimulatory capacity \( (P = 0.78) \). Concanavalin A responses are shown as a high positive control. Survival of DCs,

**FIG. 1.** A: Mass spectra of ACTH-AGE RM. The starting peptide can be seen at \( m/z = 970.5 \) and MG-H adduct at 1,025.6. B: AI-AGE RM. The starting peptide can be seen at \( m/z = 1298.05 \) and MG-H adduct at 1,352.08.

**FIG. 2.** Light scatter properties of a typical sample of AGE-exposed cells (A) and the FL4/FS histogram used to display and gate round the added count beads (B). C shows DR plus CD3 and CD19 expression in cells from the light scatter gate (R1). The DR⁺/CD3 and CD19⁻ cells (R3) were then projected onto a DR/CD14 histogram (D), and CD14⁺ and CD14⁻ populations were gated separately (R4 and R5). The proportion of CD83 expression within each gate is shown in the solid histograms (E and F). Isotype labeling (not shown) was subtracted using the WinList program.
measured by flow cytometry, was similar in AI-AGE– and buffer-exposed cultures.

**AGEs cause an increase in DC population and loss of monocytes.** PBMCs were cultured for 3 days with buffer, peptide, or AGE peptide, and aliquots were removed at various intervals. Relative numbers of DCs within all control cultures remained similar throughout culture (Fig. 7). Within AGE-exposed samples, numbers of DCs started to increase after 4 h of culture. Cells exposed to AI-AGE reached population plateau after 24 h of culture, whereas ACTH-AGE–cultured cells peaked in number at 24 h.

Monocytes within control cultures again remained similar throughout. Within the AI-AGE–exposed sample, monocyte numbers rose initially before dropping to almost zero after 24 h.

All changes were calculated relative to added count beads and thus were indicative of real changes in cell number.

**Effect of AGEs is not RAGE or CD83 mediated.** Isolated LDCs were exposed to monoclonal anti-RAGE antibody before AI-AGE RM. Whole-cell samples were prepared for analysis by MALDI-MS with blank (control) cells and cells exposed to just anti-RAGE antibody or AI-AGE RM. The use of whole cells allowed visualization of cell surface components in the absence of a large mass of intracellular components. Added AI-AGE was seen to bind quickly to the cell surface from the supernatant and was evident for several hours. Within 24 h, AI-AGE was not detectable in either the supernatant or on the cell surface. Cells exposed to anti-RAGE antibody alone looked equivalent to control cells (Fig. 8A and C). Added AGEs could be seen equivalently on the cells with and without pretreatment with anti-RAGE antibody (Fig. 8B and D). Similar results were found when blocking was performed with the anti-CD83 antibody.

PBMCs were isolated and exposed to buffer, AI, or AI-AGE RM before double-layer staining with monoclonal mouse anti-human RAGE and fluorescein isothiocyanate–conjugated polyclonal anti-mouse Ig antibodies or just anti-mouse Ig layer. Background anti-mouse Ig staining was used as a control for histogram subtraction, allowing identification of cells positive for anti-RAGE antibody. In each sample, CD14–DCs did not express RAGE, whereas a high percentage (85–91%) of the CD14– monocytes did (data not shown). Expression of RAGE in monocytes was similar in each sample.

**DISCUSSION**

This study shows for the first time that AGEs modulate the maturation and function of peripheral blood DCs and reduce their stimulatory potential. Such effects may provide a mechanistic link between build up of AGEs in diabetes and some immunological consequences of the disease.

Minimally glycated proteins are biologically significant, making it appropriate to investigate such models in vitro (2). We prepared two peptides, minimally glycated with

**FIG. 3.** Percentage expression of CD83 within the DR+/CD3 and CD19–/CD14+ population on freshly isolated PBMCs from three different donors (represented by , , and ) and the mean (represented by a bar).
MG, which had profound effects on PBMCs. We chose ACTH fragment 4–10 and AI as our starting peptides, as each contained only one arginine residue with a free amino group. This choice predicted that each peptide molecule would host only one AGE adduct, reducing complicating factors in studying its effect. The AGE adduct MG-H was detected by mass spectrometry as the major product, 54 mU above the starting peptide, within the AI-AGE reaction mixture. However, the ACTH (fragment 4–10) RM was a less simple mixture, the spectrum containing many different peaks above that of the start material, including the +54-mU peak expected; another site on this peptide may be reactive other than the considered arginine.

Both AGE peptides caused a reduction of CD83 expression in peripheral blood DCs and monocyte/macrophages, defined here as the DR+(CD3 and CD19)+/CD14+ populations, respectively. ACTH-AGE changed other surface markers, but we suggest that this was due to its toxicity, as shown by cell death.
CD83 is quoted to have an average receptor turnover time of 90 min (12) and may show effects before other surface receptors with slower recycling systems. Samples incubated for longer times, both with and without lipo-poly saccharide in the culture, showed little change in other receptors except for a lack of upregulation of CD80 after culture for 24 h. The AGE-related phenomena may not be CD83 specific.

PREVENTING SURFACE EXPRESSION OF CD83 CAN INHIBIT DC-MEDIATED T-CELL STIMULATION (22). LOSS IN THE CAPACITY OF DCs TREATED WITH AGEs TO STIMULATE PRIMARY T-CELL RESPONSES COULD BE RELATED TO THE LOSS OF BOTH CD83 AND THE COSTIMULATORY MOLECULE CD80, PARTICULARLY DURING THE CRITICAL EARLY EVENTS OF LYMPHOCYTE ACTIVATION BY DCs. AGEs DO NOT APPEAR TO BIND DIRECTLY TO CD83 AND SEEM TO BE CONSUMED BY THE CELLS, SUGGESTING THAT OTHER INDIRECT SIGNALING PATHWAYS MAY CONtribute TO FUNCTIONAL CHANGES.

Culturing peripheral blood cells with AGEs for >4 h caused an explosion in the DR+/CD3 and CD19−/CD14+ population. This effect on putative DCs was observed twice with AI-AGE and repeated once with ACTH-AGE to prove that the effect was not peptide specific. It is hard to prove a significant change in populations to account for our observations because of the small numbers of DCs compared with other cells in culture. However, known maturation of CD14+ monocytes into DCs in culture (23), together with loss of monocytes, make it likely that these are the source of the increased DC population in culture.

The most well-defined receptor for AGE is RAGE. We found that DC populations did not express RAGE, whereas monocytes expressed it highly. The effects of AGEs on DCs cannot therefore be RAGE mediated, as also suggested from the lack of blocking seen using anti-RAGE antibody in the spectrometric assay of AGE-exposed LDCs. Studies of alternative known AGE-binding sites such as macrophage scavenger receptors type I and II, galectin-3, and other scavenger receptors such as CD36, plus intracellular signaling pathways, will be required to determine the route by which the effects of AGEs are mediated.

Most long-term complications of diabetes can be related to vascular damage to either large vessels or microcirculation. Mortality associated with diabetes can be ascribed principally to large-vessel disease (24). Vessel disease in the diabetic and nondiabetic population is associated with recruitment and activation of monocytes and a subset of T-cells, with increased production of tumor necrosis factor-α, leading to raised levels of C-reactive protein and interleukin-6, both well-defined markers of inflammation (18,25,26). Specific vascular DCs are increased in number at sites of vascular damage and may modulate T-cell activation (27). Damage of vascular endothelium, leading to this inflammation, may be caused by one or more of the following factors: modified LDL, free radicals, hypertension, and infectious microorganisms, such as cytomegalovirus, Chlamydia pneumoniae, Helicobacter pylori, and herpes simplex virus (18,28,29). AGEs must now be considered as another possible factor. Although AGEs are endogenously produced products, binding to RAGE leads to a signaling cascade for activation of nuclear factor-κB and increased expression of vascular cell adhesion molecule-1 and interleukin-6 (30), which in turn cause inflammation and tissue damage. From our studies, it is unlikely that RAGE is the sole mechanism by which AGEs mediate their effects on the immune system. These long-lasting modified proteins can also stimulate a more classic immune response, because antibodies to AGEs are present in diabetic sera and correlate with related complications (31). DCs are likely to be involved in the initiation of these responses.
AGE peptide increased numbers of DCs, but these DCs did not upregulate the maturation marker CD83 or the costimulatory molecule CD80. The reduced capacity of AGE-exposed DCs to stimulate primary T-cell proliferation is compatible with the concept that the DCs remained immature in the presence of AGEs. Increased production of immature DCs that fail to mature and migrate appropriately into tissues could perhaps predispose to accumulation of such cells in vascular epithelium and consequent local innate inflammatory effects. Although we presently have no data to show a direct function of DCs in initiating such inflammation, suggestion of reduced CD83 (32), increased DC numbers (19), and impaired stimulatory capacity of DCs (20,21) in patients with diabetes support the idea that effects reported here may have some counterpart in vivo. In normal subjects, the MG-H adduct is present on up to 2% of all arginine residues (33). Levels in diabetes are likely to be higher because of raised MG concentrations. Assuming 10–20% conversion of AI to AI-AGE in our RM (judging by mass spectrum peak intensity), we can predict that addition of RM to cell culture, containing serum and other proteins, will result in a total arginine–MG-H concentration comparable to that in diabetic subjects.

In conclusion, changes to the maturation, phenotype, and function of DCs have been shown in response to AGEs. We have identified a novel pathway that may affect immunological activity in patients, particularly those with diabetes, who have high circulating levels of AGE proteins.

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