Advanced Glycosylation End Products Might Promote Atherosclerosis Through Inducing the Immune Maturation of Dendritic Cells

Junbo Ge, Qingzhe Jia, Chun Liang, Yukun Luo, Dong Huang, Aijun Sun, Keqiang Wang, Yunzeng Zou, Haozhu Chen

Objective—Both advanced glycosylation end products (AGEs) and dendritic cells (DCs) have been shown to play a causative role in atherosclerosis. However, whether they function interactively in the process remains uncertain. We therefore studied the effects of AGE–bovine serum albumin (AGE-BSA) on the maturation of DCs and the expressions of scavenger receptor-A (SR-A) and receptor for AGEs (RAGE) on DCs.

Methods and Results—AGE-BSA induced DCs maturation accompanied with increased expressions of CD1a, CD40, CD80, CD83, CD86, and MHC class II. The capacity of DCs to stimulate T-cell proliferation and secretion of cytokines (interferon [INF], INF-γ, interleukin [IL]-10 and IL-12) was also enhanced by AGE-BSA. AGE-BSA significantly upregulated SR-A and RAGE expression on DCs and the upregulation was abolished by inhibition of mitogen-activated protein (MAP) kinase Jnk, but not by that of Erk and p38 MAP kinase. AGE-BSA–induced expression of CD83 and secretion of IL-12 were partly inhibited by either an anti-RAGE neutralizing antibody or a Jnk inhibitor.

Conclusions—AGE-BSA induces maturation of DCs and augmented their capacity to stimulate T-cell proliferation and cytokine secretions possibly through upregulation of RAGE and SR-A, which at least in part through Jnk. These findings might explain in part the interactive roles of AGEs and DCs in the processes of atherosclerosis. (Arterioscler Thromb Vasc Biol. 2005;25:0-0.)

Key Words: advanced glycosylation end products □ atherosclerosis □ dendritic cells □ immunity □ receptor for advanced glycosylation end products

Diabetes is associated with severe atherosclerosis and cardiovascular diseases that account for a high morbidity and mortality in industrialized countries.1 It is therefore very important for us to understand the mechanisms of how the diseases occur. Advanced glycosylation end products (AGEs) (long-term products of the Maillard reaction) have been shown to play a causative role in diabetic vasculopathy and atherosclerosis.2,3 In diabetic atherosclerotic lesions, not only the deposition of AGEs but also the colocalization of AGEs antigen and AGEs receptors (scavenger receptor A [SR-A], receptor for AGEs [RAGE]) were found.2,3 Additionally, accumulating evidences have suggested that dendritic cells (DCs) also play a crucial role in atherosclerosis4 by activating T-cell in atherosclerosis.5 Proinflammatory factors can promote maturation of DCs corresponding to a switch from a phagocytic stage to a stage of strong T cell-stimulatory capacity. The interactive roles of AGEs and DCs remain unknown now. We therefore examined the effect of advanced glycosylation end-bovine serum albumin (AGE-BSA) on DCs maturation, cytokine secretion, and SR-A and RAGE expression.

Methods

The informed consent was obtained from all volunteers and the study protocol was approved by our institutional ethical committee.

Materials

Human CD14+ immunomagnetic microbeads were obtained from Miltenyi Biotech GmbH (Germany), recombinant human granulocyte-macrophage colony-stimulating factor (CSF) (rhGM-CSF), recombinant human IL-4 (rhIL-4), enzyme-linked immunosorbent (ELISA) kit for IL-2, IL-10, IL-12, and interferon-γ (IFN-γ) from R&D (USA), Histopaque-1077 from Sigma (USA), total RNA extraction miniprep system from Viogen-BioTEK (USA), human T-cell recovery immunocolumn kit from Cedarlane Laboratories Ltd (Canada), and SR-A (goat anti-human polyclonal antibody) from Santa Cruz Biotechnology (USA). A goat anti-human RAGE polyclonal antibody (Chemicon, USA; Cat# AB5484) was used in the present study for Western blotting. The anti-RAGE neutralizing antibody was a kind gift from the Department of Immunology, Southern Medical University (Guangzhou, China). CD1a-fluorescein isothiocyanate (FITC), CD40-FITC, CD80-FITC, CD83-FITC, CD86-FITC, HLA-DR-FITC, mouse anti-human antibodies from BD Pharmingen (USA), rabbit anti-Phospho-p38 MAP kinase antibody, rabbit anti-phospho Erk IgG, and rabbit anti-phospho-Jnk IgG from Cell Signaling Technology (USA).

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From Shanghai Institute of Cardiovascular Disease (J.G., Q.J., Y.L., D.H., A.S., K.W., Y.Z., H.C.), Zhongshan Hospital, Fudan University, Shanghai, People’s Republic of China; and the Department of Cardiology (C.L.), Changzheng Hospital, the Second Military Medical University, Shanghai, People’s Republic of China.

Correspondence to Junbo Ge, MD, Professor of Medicine, Shanghai Institute of Cardiovascular Diseases, Zhongshan Hospital, Fudan University, 180 Fenglin Rd, Shanghai 200032, P.R. China. E-mail gejunbo@zshospital.net

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Preparation of AGE-BSA
AGE-BSA was prepared as previously described. BSA was added into 10 mmol/L phosphate-buffered saline (PBS) (pH 7.4, concentration of 5 g/L), incubated with 50 mmol/L D-glucose in 5% CO2/95% air at 37°C for 12 weeks. The supernatants of DCs were harvested and stored at −70°C until use.

Preparation of Human Monocytes and Monocyte-Derived DCs
DCs were prepared as previously described. Peripheral blood mononuclear cells were obtained from healthy volunteers. Briefly, blood was diluted 1:2 in PBS layered over Histopaque 1077 and centrifuged for 30 minutes at 2000 rpm at room temperature. The interface was recovered and washed 3 times in PBS. CD14+ peripheral blood mononuclear cells were purified by using CD14+ immunomagnetic micro beads (>98%) and incubated in RPMI-1640 medium supplemented with GM-CSF (100 ng/mL) and IL-4 (50 ng/mL) in 6-well tissue culture plates at 37°C and an atmosphere of 5% CO2. The medium was replaced every 2 days. After 8 days, DCs were stimulated by AGEs.

Semi-Quantitative Reverse-Transcription PCR Analysis
Total cellular RNA was extracted from cells using total RNA extraction miniprep system according to the manufacturer’s instruction. Oligo (dT)18 were used for cDNA preparation. For detection of SR-A, the primers were as follows (365 bp): forward, 5'-CCAGGGACATGGGAATGCAA-3'; reverse, 5'-CCAGTGGG-ACCTCGATCTCC-3'. Primers for RAGE were as follows (240bp): forward, 5'-AAGCCCTCTTGCCTAATGAG-3'; reverse, 5'-CCAAATTGGACCTCCTCCA-3'. A value of p<0.05 was considered statistically significant.

Western Blot Analysis
Cells were lysed in RIPA plus PMSF at 4°C with sonication for 30 minutes. The lysates were centrifuged at 15,000g for 30 minutes. Loading buffer was added to each volume and boiled for 10 minutes. The lysates were centrifuged at 15,000g for 30 minutes. The supernatants of DCs were harvested and stored at −70°C until use.

Flow Cytometric Measurement
DCs were exposed to 0 μg/mL AGE-BSA, 200 μg/mL BSA, or 200 μg/mL AGE-BSA for 24 hours. Cells (1×10^6) were harvested and washed, then incubated with FITC-conjugated mAbs (CD1a-FITC, CD40-FITC, CD80-FITC, CD83-FITC, CD86-FITC, HLA-DR-FITC) for 30 minutes at 4°C. Then, the cells were analyzed using flow cytometer (Becton Dickinson). Cells stained with the appropriate isotype-matched Ig were used as negative controls.

Quantification of Cytokine Production
The supernatants of DCs were harvested and stored at −70°C until use. The cytokine concentration of IL-2, IL-10, IL-12, and IFN-γ were analyzed using ELISA kits, according to the manufacturer’s instruction.

Mixed Lymphocyte Reactions
For the mixed lymphocyte reaction (MLR), T lymphocytes were isolated from peripheral blood by human T cell recovery immuno-column kit. MLRs were conducted in 96-well flat-bottom culture plates. DCs were treated by BSA (200 μg/mL) or AGE-BSA (200 μg/mL) for 24 hours. Untreated DCs were as control. These cells were then cultured with 2×10^5 T cells in 200 μL complete culture medium at 1:5, 1:10, and 1:20 DC:T cell ratio. Before mixed, DCs were pretreated by mitomycin (150 μmol/L) for 1 hour at 37°C. After 96 hours, 50 μL culture supernatants were collected, stored at −70°C, and analyzed by ELISA for production of IL-2, which is a marker of T-cell activation. Then, 50 μL fresh medium containing 1 μCi of [3H] thymidine were added. After 16 hours, the cells were harvested onto filter paper, and the incorporation of [3H] thymidine was determined by scintillation counting. Experiments were performed in triplicate.

Statistical Analysis
All data were expressed as mean±SEM and statistically analyzed by 1-way ANOVA. A value of p<0.05 was considered statistically significant.

Results
Dendritic Cells Morphology
The CD14 microbeads sorted monocytes were symmetrical and the purity was >98%. Twenty-four hours later, the most cells formed the different clones, which became large; after 96 hours, the dendrites were clearly observed (data not shown).

Concentration-Dependent Upregulation of SR-A Expression in DCs by AGE-BSA
DCs were exposed to BSA (200 μg/mL) or AGE-BSA at various concentrations (0, 50, 100, 200, and 300 μg/mL) for 24 hours. SR-A expression of DCs at mRNA and protein level after various treatments were shown in Figure 1A and 1B, respectively. SR-A at mRNA and protein levels increased in a concentration-dependent manner post AGES treatment and peaked at a concentration of 200 μg/mL, whereas SR-A expression remained unchanged in BSA-treated DCs.

Time-Dependent Upregulation of SR-A Expression in DCs by AGE-BSA
DCs were exposed to AGE-BSA (200 μg/mL) for 0, 6, 12, 24, and 36 hours. SR-A expressions of DCs at mRNA and protein levels after various treatments were shown in Figure 1C and 1D, respectively. SR-A expressions were increased at both mRNA and protein levels in a time-dependent manner.

RAGE Expression in AGE-BSA–Treated DCs
RAGE expressions at mRNA and protein levels at control condition, treated with BSA (200 μg/mL) or AGE-BSA (200 μg/mL) for 24 hours were analyzed by Western blotting. RAGE expression was upregulated by AGE-BSA but not by BSA treatment. The expression was time-dependent and peaked at a concentration of 200 μg/mL, whereas RAGE expression remained unchanged in BSA-treated DCs.
AGE-BSA Stimulation Activates MAPK Pathways in DCs

Phosphorylation of MAP kinase family proteins including Erk, Jnk, and p38 was measured to gain insight into the changes of AGE-BSA–induced signaling pathways in DCs. DCs were stimulated with AGE-BSA (200 μg/mL) for 0, 10, 20, 30, and 40 minutes. Phosphorylation of Erk, Jnk, and p38 MAPK was assayed by Western-blot. Increases in the phosphorylation levels of Erk, Jnk, and p38 were visible at 10 minutes, peaked at 20 minutes, and declined thereafter (Figure 2A through 2C).

Role for MAPK Pathways in the AGE-BSA–Induced Upregulation of the SR-A and RAGE

DCs were exposed to AGE-BSA (200 μg/mL), AGE-BSA (200 μg/mL) plus Erk inhibitor PD98059 (50 μmol/L),11 AGE-BSA (200 μg/mL) plus Jnk inhibitor SP600125 (50 μmol/L),12 and AGE-BSA (200 μg/mL) plus p38 MAPK inhibitor SB203580 (10 μmol/L).11 DCs were pretreated with respective inhibitors for 1 hour, followed by stimulation with AGE-BSA for an additional 23 hours, and the expressions of SR-A and RAGE were measured. The increase in SR-A and RAGE expressions after AGE-BSA was significantly inhibited by cotreatment with Jnk inhibitor SP600125, but not by Erk inhibitor PD98059 and p38 MAPK inhibitor SB203580 (Figure 2D and 2E).

AGE-BSA–Induced Upregulation of Surface Molecules in DCs

Fluorescence-activated cell sorter (FACS) analysis showed that the expressions of CD1a, CD40, CD80, CD83, CD86, and HLA-DR in AGE-BSA–treated DCs were increased significantly compared with the BSA and untreated DCs (Figure 3). It indicated that AGE-BSA could promote the maturation of DCs.

AGE-BSA–Induced Cytokine Secretions by DCs

DCs were exposed to BSA (200 μg/mL) or AGE-BSA (200 μg/mL). After AGE-BSA treatment, secretion of IFN-γ (Figure 4A), Th1 cytokine IL-12 (Figure 4B), and Th2 cytokine IL-10 (Figure 4C) were all increased compared with BSA treated DCs.
and untreated control DCs. However, IL-2 levels remained unchanged after AGE-BSA treatment (Figure 4D).

**AGE-BSA Enhances the Capacity of DCs to Stimulate T Cells**

Compared with untreated control DCs, AGE-BSA–treated DCs increased the proliferation of T cells (Figure 5A). Simultaneously, AGE-BSA–treated DCs increased the secretion of IL-2 by T cells by 3-fold at 1:5 and 1:10 DCs/T cells ratio (Figure 5D and 5C) and by 2-fold at 1:20 DCs/T cells ratio (Figure 5B).

**Role of Anti-RAGE Neutralizing Antibody in the AGE-BSA–Induced Upregulation of CD83 and Secretion of IL-12**

DCs were pretreated with 50 μg/mL of anti-RAGE neutralizing antibody for 1 hour followed by 200 μg/mL of AGE-BSA for 23 hours. Untreated DCs were as control. Compared with AGE-BSA–treated DCs, anti-RAGE neutralizing antibody inhibited partly upregulation of CD83 and secretion of IL-12 (Figure 6A and 6C).

**Role of Jnk Inhibitor in the AGE-BSA–Induced Upregulation of CD83 and Secretion of IL-12**

DCs were pretreated with Jnk inhibitor SP600125 (50 μmol/L) for 1 hour followed by 200 μg/mL of AGE-BSA for 23 hours. Untreated DCs were as control. Compared with AGE-BSA–treated DCs, SP600125 inhibited upregulation of CD83 and secretion of IL-12 (Figure 6B and 6D).

**Discussion**

In the present study, we found that AGE-BSA induced the maturation of DCs characterized by upregulation of CD1a, CD40, CD80, CD83, CD86, and MHC class II. In parallel, secretion of proinflammatory or Th1-type cytokines, like IFN-γ and IL-12, and also the Th2-type cytokine IL-10, was significantly increased after AGE-BSA treatment. In addition, AGE-BSA enhanced the capacity of DCs to stimulate T-cell proliferation in allogenic MLRs. Moreover, AGE-BSA enhanced the expressions of SR-A and RAGE, and the inhibition of the receptor expression abolished AGE-BSA–induced DCs maturation and cytokine secretion. Furthermore, inhibition of Jnk suppressed upregulation of not only SR-A

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**Figure 2.** Effect of AGE-BSA on phosphorylation of MAPK and expression of SR-A and RAGE in the presence of specific inhibitors for MAPK family, A, B, C, *P*<0.05 vs 0 minutes. Mean±SEM. n=3. D and E, Control (lane 1), 200 μg/mL AGE-BSA (lane 2), 200 μg/mL AGE-BSA plus 50 μmol/L SP600125, Jnk inhibitor (lane 3), 200 μg/mL AGE-BSA plus 50 μmol/L PD98059, Erk inhibitor (lane 4), and 200 μg/mL AGE-BSA plus 10 μmol/L SB203580, p38 MAPK inhibitor (lane 5). The proteins of SR-A (D) and RAGE (E) were extracted from DCs and analyzed by Western blot. *P*<0.05 vs 0 μg/mL AGE-BSA; †P<0.05 vs 200 μg/mL AGE-BSA. Mean±SEM. n=3.

**Figure 3.** The immunophenotypic expressions of DCs exposed to AGE-BSA. Control (green), 200 μg/mL BSA (red), and 200 μg/mL AGE-BSA (blue). Flow cytometric analysis was performed for surface molecule expression examination.
and RAGE expressions on DCs but also DCs maturation and cytokine secretion induced by AGE-BSA. Therefore, AGE-BSA could be involved in atherosclerosis processes through inducing DCs maturation and increasing SR-A and RAGE expressions, which were mediated by Jnk signal pathway. To our knowledge, this is the first study showing these important effects of AGEs on DCs.

Previous immunohistochemical and ultrastructural studies have shown increased DCs in atherosclerotic arteries.\textsuperscript{13–15} Various antigens, such as oxidized low-density lipoprotein (ox-LDL),\textsuperscript{16} heat shock protein (HSP),\textsuperscript{17} and nicotine,\textsuperscript{11} could activate DCs immunity. The present study showed for the first time that AGEs could also induce the maturation of DCs. Compared with immature DCs, maturation of DCs involves the downregulation of endocytotic activity and the upregulation of adhesion molecules, costimulatory molecules, and antigen-presenting molecules, including the class I and class II of MHC proteins and CD1 molecules. Coexpression of the adhesion and costimulatory and antigen-presenting molecules enable DCs to form contacts with and activate T cells. In our study, we found AGE-BSA enhanced the expressions of CD1a, CD40, CD80, CD83, CD86, and HLA-DR. Therefore, AGE-BSA could also induce the maturation of DCs like ox-LDL. Moreover, we found that AGE-BSA–treated DCs were able to secrete more IL-12 than IL-10. This finding suggested that AGE-treated DCs might be more potent in priming naive T cells for type-1 cytokine response and cell-mediated immunity. Secretion of IFN-γ from DCs was also increased by AGE-BSA. Because IL-12 and IFN-γ released by activated DCs contributed to the formation of atherosclerotic lesions and plaque destabilization,\textsuperscript{18,19} our findings suggest that AGEs might promote the atherosclerosis by enhancing the maturation and immunity of DCs. Quite recently, Price et al\textsuperscript{20} reported that AGEs prohibited the maturation of DCs and AGE-treated DCs had a loss in T-cell stimulation capacity, which is different from our present study. Although we do not know the reason for the discrepancy at present, there might be several possibilities. At first, Price et al used CD14\textsuperscript{−} DCs whereas we used CD14\textsuperscript{+} DCs.
The difference in cell types might cause different results. Whether CD14− and CD14+ DCs function differently in atherosclerosis remains unclear. Second, Price et al used AGE-corticotropin (ACTH) angiotensin, whereas we used AGE-BSA as an antigen. The responses of DCs to the 2 antigens might be divergent.

It was found that IL-2 further activated the T cells and might potentially enhance atherogenesis. In this study, we did not find a significant increase of IL-2 in DCs by AGE-BSA treatment; however, in the presence of T cells (MLRs), AGE-BSA–treated DCs strongly stimulated the proliferation of T cells and the secretion of IL-2 in proportion to DCs/T ratio. Therefore, IL-2 was mainly originated from T cells and AGEs could further magnify the effects of IL-2 in atherosclerosis by activating the T cells and increasing IL-2 secretion via DCs.

AGEs have currently been shown to play a causative role in diabetic vasculopathy, including atherosclerosis as well as microangiopathy. Previous mechanism studies in this field were mainly focused on endothelial cells, monocyte cells, and smooth muscle cells. Results of the present study show that one of the mechanisms in which AGEs promote atherosclerosis is achieved by their effects on DCs. SR-A is one of the receptors of AGEs and responsible for mediating cholesterol accumulation by macrophages during the development of atherosclerotic lesions. A recent study has shown that the atherosclerotic lesions were reduced by more than 70% in SR-A knockout mice. In accordance with this finding, we found that AGEs could enhance the expression of SR-A in DCs in dose and time dependent manners, supporting the role of AGEs on atherogenesis.

RAGE is the specific receptor of AGEs. RAGE is expressed at low levels in normal tissues and vasculature. In diabetic vasculature, cells expressing high levels of RAGE are often proximal to areas in which AGEs are abundant. RAGE expression is increased in endothelium, smooth muscle cells, and infiltrating mononuclear phagocytes in diabetic vasculature. In this study we found AGE-BSA could significantly enhance the expression of RAGE and activate the MAPK family (Erk, Jnk, P38) in DCs. Previous studies showed that Erk and Jnk inhibition protected various cells from apoptosis and both Erk and p38 MAPK could upregulate surface molecules. The Jnk inhibitor, but not Erk and p38 inhibitors, significantly attenuated the expression of RAGE and SR-A. It suggested the possible involvement of Jnk pathway in AGE-BSA–induced upregulation of SR-A and RAGE expressions. In addition, we also observed that pretreatment of DCs with an anti-RAGE neutralizing antibody or a Jnk inhibitor partly inhibited AGE-induced upregulation of CD83 and secretion of IL-12. It suggests that AGE-induced maturation of DCs is at least in part through RAGE and Jnk signal pathway. Combining with the result that Jnk inhibitor attenuated the induction of RAGE and SR-A on DCs, we suggest that Jnk inhibitor decreased the amount of AGEs into DCs, resulting in the decrease of the antigen in DCs and then the lesser DC maturation and cytokine secretion. However, as we tried to test whether SR-A is also involved in the effects of AGE on DCs by using 2 SR-A inhibitors (namely genistein and name polyinosinic acid), it was found that the treatment with either of them affected maturation of DCs. Polyinosinic acid could induce the maturation of DCs and Genistein could inhibit directly the maturation of DCs because it was an inhibitor of tyrosine kinase. These results failed to provide direct evidence showing whether SR-A is involved in the receptor induction and DC maturation by AGE. However, because the Jnk inhibitor that suppressed both RAGE and SR-A expressions on DCs had a stronger inhibitory effect on DCs maturation than an anti-RAGE neutralizing antibody, which only partially inhibited DCs maturation, it may be suggested that AGE-BSA entered DCs and induced DC maturation through both RAGE and SR-A. The sequelae of this increased expression of RAGE and SRA by AGEs might modulate inflammatory response by increasing the expression of oxidant radical formation, proinflammatory cytokines, growth factors, and vascular adhesion molecules, therefore promoting atherosclerotic plaque formation, endothelial dysfunction, and diabetic vasculopathy.

Our results suggest that AGEs bind to DCs through SR-A and RAGE receptors, induce the maturation of DCs, and activate antigen-specific T cells, as well as initiate immune reaction and promote atherosclerosis. Our recent study showed that PPARγ agonist ciglitazone could inhibit the ox-LDL–induced maturation of DCs. It is, therefore, reasonable to speculate that AGEs inhibitors might be also candidates for atherosclerosis therapy by inhibiting DCs maturation and immunity.

In conclusion, AGEs could induce the maturation of DCs and enhance immunity of DCs through increasing the expressions of SR-A and RAGE, which were mediated by Jnk pathway. The present study suggests a possibility of the application of anti-RAGE antibody and Jnk inhibitors for atherosclerosis therapy.

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