Advanced glycation endproducts alter functions and promote apoptosis in endothelial progenitor cells through receptor for advanced glycation endproducts mediate overpression of cell oxidant stress

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Abstract Endothelial progenitor cells (EPCs) play an important role in preventing atherosclerosis. The factors that regulate the function of EPCs are not completely clear. Increased formation of advanced glycation endproducts (AGES) is generally regarded as one of the main mechanisms responsible for vascular damage in patients with diabetes and atherosclerosis. AGEs lead to the generation of reactive oxygen species (ROS) and part of the regenerative capacity of EPCs seems to be due to their low baseline ROS levels and reduced sensitivity to ROS-induced cell apoptosis. Therefore, we tested the hypothesis that AGEs can alter functions and promote apoptosis in EPCs through overpress cell oxidant stress. EPCs, isolated from bone marrow, were cultured in the absence or presence of AGEs (50, 100, and 200 μg/ml). A modified Boyden’s chamber was used to assess the migration of EPCs and the number of recultured EPCs was counted to measure the adhesiveness function. MTT assay was used to determine the proliferation function. ROS were analyzed using the ROS assay kit. A spectrophotometer was used to assess superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX) activity, and PCR was used to test mRNA expression of SOD and GSH-PX. SiRNA was used to block receptor for advanced glycation endproducts (RAGEs) expression. Apoptosis was evaluated by Annexin V immunostaining and TUNEL staining. Co-culturing with AGEs increases ROS production, decreases anti-oxidant defenses, overpresses oxidant stress, inhibits the proliferation, migration, and adhesion of EPCs, and induces EPCs apoptosis. In addition, these effects were attenuated during block RAGE protein expression by siRNA. AGEs may serve to impair EPCs functions through RAGE-mediate oxidant stress, and promote EPCs sensitivity toward oxidative-stress-mediated apoptosis, which indicates a new pathophysiological mechanism of disturbed vascular adaptation in atherosclerosis and suggests that lower levels of AGEs might improve the success of progenitor cell therapy.

Keywords Advanced glycation endproducts · Receptor for advanced glycation endproducts · Endothelial progenitor cells · Atherosclerosis · Oxidative stress · Apoptosis

Introduction

Diabetes-associated cardiovascular complications are one of the major causes of patient mortality [1]. Numerous epidemiological studies suggest that diabetes can accelerate atherosclerosis and increase the incidence of heart attacks and strokes. However, the underlying mechanisms behind this relationship have not been fully elucidated. Advanced glycation endproducts (AGEs) are produced by the post-translational modification of proteins via non-enzymatic glycation. Increased formation of AGEs is generally regarded as one of the main mechanisms responsible for vascular damage in patients with diabetes. AGEs cross-linking of peptides and proteins is protease-resistant and causes irreversible damage to tissues and activation of macrophages, monocytes, cardiac fibroblasts, and vascular smooth muscle cells. Activation of these cells leads to the generation of reactive oxygen species (ROS), which induces DNA oxidation and membrane lipid peroxidation [2–4].
Increasing evidence indicates that AGEs-induced oxidative stress has a role in the pathogenesis of normal aging and many age-related chronic diseases, including atherosclerosis and diabetes [5–8]. Furthermore, the pathophysiological significance of AGEs induce oxidative stress is supported by animal and clinical studies showing improved arterial compliance after blocking receptor for advanced glycation endproduct (RAGE), a multiligand cell surface receptor.

Endothelial progenitor cells (EPCs) are bone-marrow-derived stem cells that have the ability to proliferate, migrate, and differentiate into functional, mature endothelial cells. EPCs circulate in the blood and seem to preferentially localize to sites of vascular or tissue injury, contributing significantly to both re-endothelialization and neovascularization, which are functionally important in vascular repair [9–12]. Part of the regenerative capacity of EPCs seems to be due to their low baseline ROS levels and reduced sensitivity to ROS-induced cell apoptosis [13, 14]. Diabetes contributes to reduction and dysfunction of circulating progenitor cells [15, 16]. And the higher concentration of AGEs could be the reason. Atherosclerosis is currently regarded as a dynamic and progressive disease arising from the combination of endothelial dysfunction and inflammation. EPCs circulate in the blood and play an important role in the formation of new blood vessels as well as contribute to vascular homeostasis in the adult. Numerous studies have shown that EPCs can integrate into new and existing blood vessels and repair endothelial dysfunction.

Owing to the critical role of EPCs in maintaining endothelial function by contributing to re-endothelialization and neovascularization, a disadvantageous effect of AGEs on EPCs function may account for the progression of vascular complications in atherosclerosis. The purpose of this study is to examine whether AGEs can influence EPCs and determine the mechanism involved.

**Materials and methods**

**Cells isolation and culture**

EPCs were cultured according to previously described techniques [10]. Briefly, total mononuclear cells (MNCs) were isolated from the bone marrow of Sprague–Dawley rats (150–160 g, n = 20) by density-gradient centrifugation at 500×g for 20 min and washed three times with phosphate-buffered saline (PBS) (Jingmei BioTech Co. Ltd). The collected MNCs were then seeded on 24-well plates (10⁶ cells/well) in DMEM (Hyclone) with 20% fetal calf serum (Hyclone), 100-U/ml penicillin, 100-U/ml streptomycin, and vascular endothelial growth factor (VEGF, 50 ng/ml). Four days after culture, non-adherent cells were abandoned. Adherent cells were cultured for 7 days.

**Cells staining and selection**

Adherent cells were first incubated with 2.4-μg/ml Dil-labeled acetylated low-density lipoprotein (LDL) (Molecular Probe) at 37°C for 12 h and then fixed with 2% paraformaldehyde for 10 min. After washing with PBS, the cells were incubated with 10-μg/ml FITC-UEA-I (Vector) at 37°C for 1 h. After staining, samples were viewed with an inverted fluorescence microscope (Leica) and also with laser confocal microscopy (LSCM, Leica). Cells demonstrating double-positive fluorescence were identified as differentiating EPCs. Two or three independent investigators evaluated the number of EPCs per well by counting 15 randomly selected high-power fields [17]. In addition, adherent cells were detached with 0.25% trypsin followed by repeated gentle flushing through a pipette tip and were then washed in PBS. 1 × 10⁵ cells were incubated with anti-CD133 (Biosynthesis BioTech Co. Ltd) and anti-VEGFR-2-FITC (eBiosciences) in the dark for 30 min at room temperature, as well as their corresponding isotype controls. After that, flow-cytometric analyses were performed using a FACScan flow cytometer and Cell Quest software (BD Biosciences).

**Cell incubation**

Bone-marrow-derived EPCs were cultured for 7 days, then cells were incubated with various concentrations of AGEs (Jingmei BioTech Co. Ltd) (0, 50, 100, 150, 200 and 400 μg/ml) for 24 h.

**Detection the protein expression of RAGE**

The effect of AGEs on the protein expression of RAGE was determined by Western blot. Rabbit anti-rat RAGE antibody (Boisynthesis BioTech Co. Ltd, 1:50) was used as primary antibody and the goat anti-rabbit IgG-horseradish peroxidase secondary antibody (Beyotime Institute of Biotechnology). Briefly, EPCs lysates were fractionated through a 5% stacking and 10% resolving SDS-PAGE, gel and the fractionated proteins were transferred to nitrocellulose membrane. Blots were blocked for 1 h at room temperature with blocking buffer [5% non-fat milk in 10-mmol/l Tris, pH 7.5; 100-mmol/l NaCl, 0.1% Tween 20]. Primary antibody, at a dilution of 1:500, was reacted with the blots overnight at 4°C. After washing three times for 5 min in 1× Tris-buffered saline with 0.1% Tween 20, the blots were incubated with the secondary antibody at a dilution of 1:1000 for 1 h at room temperature. To ensure
equal loading of intact protein, membranes were stripped and restained with antibodies against GAPDH.

Small interfering RNA assay

Small interfering RNA (siRNA) and transfection reagent for RAGE were purchased from Ambion Inc. SiRNA transfection was carried out according to the manufacturer’s protocol for the RAGE–siRNA kit. In brief, 1 × 10^6 EPCs were transferred into 24-well plates and cultured in DMEM supplemented with 20% fetal calf serum, without antibiotics. After overnight growth, cells at 80% adherent were treated with 0.5 ml of Opti-MEM I (Invitrogen Corporation) with each well containing 100-nmol/l siRNA duplexes and 5 μl of lipofectamine2000. In addition, Cy3-labeled negative control siRNA were used to detect the transfection efficiency. After 48 h of incubation, cells transplanted with Cy3-labeled siRNA were collected to detect the transfection efficiency.

ROS analysis

ROS formation was measured by the detection of H2DCF-DA (Sigma) by flow-cytometry analysis following the manufacturer’s instructions. Isolated EPCs were detached using 0.25% trypsin, harvested by centrifugation, resuspended in 500-μl DMEM, and counted. 1 × 10^5 cells and 1 μl of H2DCF-DA were mixed and incubated in the dark for 15 min at room temperature. The mean fluorescence intensity of cells for each sample was quantified. Unstained cells were used as controls.

Detection of anti-oxidative enzymes

To examine whether AGEs interfere with the expression of anti-oxidative enzymes in EPCs, levels of superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX) expression were test by RT-PCR. Total cellular RNA was isolated using the RNeasy Mini kit (Tiandz). RT-PCR was performed using the RT-PCR kit (TOYOBO BioTech Co. Ltd). Total RNA served as template for each reaction. For amplification, a primer pair specific was used. The primers used for rat SOD were the following: forward: 5′-CTGCAGGACCTCATTTT-3′; and reverse: 5′-CACCTTGCCCAAGTCACTCT-3′. The primers used for rat GSH-PX were the following: forward: 5′-GTCCACGGTGATG CCTTCT-3′; and reverse: 5′-CATTACCTCGACCTTC TCA-3′. Reverse transcription was performed at 42°C for 20 min, 99°C for 5 min, 5°C for 5 min. For PCR, 30 cycles were used at 94°C for 3 min, 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s. The RT-PCR products were visualized on 1% agarose gels using ethidium bromide. GAPDH was amplified as a reference for quantitation mRNA. And the activity of SOD and GSH-PX were assessed by spectrophotometry analysis; as the optical density increases, the enzymes activity level reduces. Each A value was measured at 550 and 412 nm.

Migration assay

EPCs migration was evaluated using a modified Boyden chamber as previously described. Isolated EPCs were detached using 0.25% trypsin, harvested by centrifugation, resuspended in 500-μl DMEM, and counted. A total of 2 × 10^5 EPCs were placed in the upper chamber of a modified Boyden’s chamber (Qilin instrument, Jiangsu province China). After 24-h incubation at 37°C, the lower side of the filter was washed with PBS and fixed with 2% paraformaldehyde. For quantification, cell nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI). Cells migrating into the lower chamber were counted manually in every three random microscopic fields (×400).

Adhesiveness assay

A total of 1 × 10^6 EPCs were placed on 24-well plates in DMEM at 37°C for 1 h. The number of recultured EPCs was counted manually in three random microscopic fields (×400) to assess adherent function [17].

Proliferation assay

Mitogenic activity was assayed as follows: primarily cultured EPCs were trypsinized with 0.25% trypsin, washed with PBS, and suspended in DMEM, 5 × 10^5 cells/well were plated on fibronectin-coated 96-well plates. After incubation for 24 h, the medium was replaced and kept on growing in incubator. 48 h later, the EPCs proliferation was tested. Before the optical density measurement (490 nm) was performed, EPCs were supplemented with 15-μl MTT (5 g/l) for another 5 h and 200-μl dimethyl sulfoxide (DMSO) for 10 min, respectively.

Detection of apoptosis

Entry into apoptosis leads to the translocation of phosphatidyl serine from the inner leaflet to the extracellular side of the plasma membrane. Terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) is a common method for detecting DNA fragmentation resulting from apoptotic signaling cascades. The assay involves detection of nicks in the DNA by terminal deoxynucleotidyl transferase, an enzyme that will catalyze the addition of dUTPs that are secondarily labeled with a marker. Also, annexin V, a protein that binds to phosphatidyl serine with high affinity, can be used to detect apoptosis-induced membrane changes.
By simultaneously staining cells with propidium iodide (PI), it is possible to discriminate between apoptotic, necrotic, and viable cells in a cell suspension. The TUNEL kit and Annexin-V-Fluos kit were obtained from Jingmei BioTech Co. Ltd.

Statistical analysis

For statistical analysis, standard methods were used. Data are given as mean ± SEM. Differences between groups were assessed by an independent-sample t test for single comparisons and by ANOVA for multiple comparisons. \( P < 0.05 \) was considered significant.

**Results**

Characterization of bone-marrow-derived EPCs

Bone-marrow-derived MNCs were cultured in endothelial cell selection medium for 7 days. Attached cells turned into spindle-shaped cells (Fig. 1a), and 88.7 ± 4.8% of them showed uptake of DiI-acLDL and FITC-UEA-I, demonstrating endothelial cell characteristics (Fig. 1b). These cells were characterized further by demonstrating expression of the rat stem cell marker CD133 (83.7 ± 4.3%) and the endothelial cell lineage antigen VEGFR-2 (70.2 ± 3.5%) by flow-cytometry analysis (n = 10) (Fig. 1c).

**Fig. 1** Bone-marrow-derived MNCs differentiate into cells with EPC characteristics after in vitro expansion. a After 7 days culture, bone-marrow-derived MNCs exhibited spindle-shape morphology, similar to endothelial cells (×400). b Bone-marrow-derived MNCs show uptake of acetylated low-density lipoprotein (LDL) (b1 red) and FITC-UEA-I (b2 green). Most adherent cells (88.7 ± 4.8%; n = 10) are double-positive (b3 yellow). (×200). c Most adherent cells express the VEGFR-2 antigen (70.2 ± 3.5% n = 10), as shown by flow-cytometry analysis (Color figure online)
AGEs increase RAGE protein expression in a dose-dependent manner

To examine whether AGEs affect RAGE expression, EPCs were treated with AGEs at different doses, and RAGE protein was quantified using Western blotting. EPCs were treated with AGEs at a dose from 50 to 400 µg/ml for 24 h. Results from Western blot analysis revealed a stepwise increase in RAGE protein expression with increasing AGEs concentration. RAGE protein levels were significantly induced by 33, 59, 80, 97, and 114% in response to AGEs concentrations of 50, 100, 150, 200, and 400 µg/ml, respectively, when compared with controls (Fig. 2a, b).

Blocking RAGE protein expression by siRNA

SiRNA was used to block RAGE expression. And Cy3-labeled negative control siRNA were used to detect the transfection efficiency. After 48 h of incubation, cells transplanted with Cy3-labeled siRNA were detected by fluorescence microscope (Fig. 3).

Effects of AGEs on the formation of ROS

ROS formation was measured by detection of H$_2$DCF-DA by flow-cytometry analysis. Treatment of EPCs with AGEs significantly increased the generation of ROS in a dose-

![Fig. 2](image1.png)

**Fig. 2** Effects of different concentrations of AGEs on RAGE protein expression. EPCs were cultured for 24 h with AGEs at a dose of 50–400 µg/ml. Protein levels were assessed by Western blot (a). AGEs up-regulates RAGE protein expression in a dose-dependent manner (b). Significances of differences are as indicated: $n = 5$, $^* P < 0.01$ group comparison

![Fig. 4](image2.png)

**Fig. 4** Effects of different concentrations of AGEs on ROS production. AGEs can increase ROS production. The fluorescence intensity of ROS increased with increasing AGEs concentrations; however, the effect was attenuated during blocked RAGE by siRNA. Significances of differences are as indicated: $n = 5$, $^* P < 0.01$ group comparison, $^{**} P < 0.01$ versus AGEs 400

![Fig. 3](image3.png)

**Fig. 3** Pre-designed siRNA was transfected into EPCs through Lipofectamine 2000 to silence RAGE. And Cy3-labeled negative control siRNA was used to detect the transfection efficiency. After 48 h of incubation, cells transplanted with Cy3-labeled siRNA were detected by fluorescence microscope. Most of the EPCs show uptake of Lipofectamine 2000 and display red fluorescence in cytoplasm. (×200) (Color figure online)
dependent manner; however, the effect was attenuated during silenced RAGE by siRNA (n = 5, P < 0.01) (Fig. 4a, b).

Effect of AGEs on anti-oxidative enzymes

The mRNA levels of SOD and GSH-PX expression were tested by PCR. AGEs treatment significantly decreased SOD mRNA levels to 81.2, 71, 51, 44.2, and 42.4% in response to AGEs concentrations of 50, 100, 150, 200, and 400 μg/ml, respectively, when compared with controls. Silenced RAGE can obstruct this effect (n = 5, P < 0.01) (Fig. 5a). GSH-PX mRNA levels were significantly decreased to 80.4, 68.6, 53.0, 40.6, and 37.4%, and silenced RAGE can obstruct this effect (n = 5, P < 0.01) (Fig. 5b). And the activity of SOD and GSH-PX were determined by spectrophotometry analysis; the optical density increases as the enzyme activity levels decrease. As shown in Fig. 5c, d, AGEs treatment significantly increased the optical density of anti-oxidative enzymes (n = 5, P < 0.05). Interestingly, silenced RAGE also can obstruct this effect.

Effect of AGEs on EPCs migration

Migrate function was determined using a modified Boyden’s chamber. In this study, we investigated the effect of AGEs on EPC migration. Data revealed that the migration function of EPCs was significantly reduced with higher AGEs concentrations. In addition, silenced RAGE can attenuate this effect (n = 10, P < 0.01) (Fig. 6).

Effect of AGEs on EPC adhesiveness

We also evaluated the effects of AGEs on EPC adhesiveness. After being replated on fibronectin-coated dishes, EPCs pre-exposed to AGEs exhibited a significant dose-dependent reduction in the number of adherent cells. However, this reduction can be obstructed by silenced RAGE (n = 10, P < 0.01) (Fig. 7).

Effect of AGEs on EPCs proliferation

Results from the MTT assay showed that AGEs inhibited the proliferative activity of bone-marrow-derived EPCs in

![Fig. 5](https://example.com/fig5.jpg)

**Fig. 5** Effect of AGEs on anti-oxidative enzymes. a AGEs treat can decrease SOD mRNA expression. b AGEs treat can decrease GSH-PX mRNA expression. * P < 0.01 group comparison, ** P < 0.01 versus AGEs 400. c, d AGEs treat increased the optical density. Blocking RAGE can obstruct this effect. Significances of differences are as indicated: n = 5, * P < 0.05 group comparison, ** P < 0.01 versus AGEs 400.
a dose-dependent manner. Silenced RAGE can also block this effect (n = 10, P < 0.01) (Fig. 8).

Discussion

Diabetes and its observed ability to accelerate the progression of atherosclerosis cannot simply be explained by the traditional cardiac risk factors, such as smoking, hypertension, and hyperlipidemia [18]. Recently, AGEs was identified as one of the underlying mechanisms that may assist in explaining this observation. The interaction of AGEs with specific cell receptors leads to cellular activation, increased expression of extracellular matrix proteins and the release of proinflammatory cytokines and ROS. AGE-binding receptors include AGE-R1, AGE-R2, AGER3, the scavenger receptor II and receptor for AGEs (RAGE). AGE-R1, formerly termed oligosaccharyltransferase 48 (OST48), is a 48 kDa endocytosis-mediating protein. AGE-R2 is involved in signal transduction via protein kinase C. AGER3 (galectin-3) can influence cell growth, adhesion, differentiation, apoptosis, and malignant transformation. The scavenger receptor II are probably implicated in the removal of AGEs. RAGE is the best characterized AGE receptor and responsible for most of the deleterious effects of AGEs. As a multiligand receptor on vascular cells, RAGE has been shown to promote atherogenesis [19, 20]. Atherosclerosis is becoming an important cause of death due to the increasing elderly population. Endothelial injury and dysfunction have important roles in atherosclerotic processes. As bone-marrow-derived EPCs contribute to re-endothelialization and neovascularization, increasing the function of these cells may be an attractive therapeutic tool. AGEs levels are elevated in atherosclerosis and EPCs functions are impaired in atherosclerosis, suggesting that these two factors are involved in its progression. However, it is not entirely clear as to whether there is an interaction between these two factors. The
The purpose of our study is to investigate the effects of AGEs on bone-marrow-derived EPCs. In this study, we first demonstrated that AGEs promote generation of ROS in EPCs in a dose-dependent manner. Second, the anti-oxidase mRNA level and activity level are decreased by AGEs treatment in a dose-dependent manner. Third, the functional activities of EPCs are impaired by AGEs also in a dose-dependent manner and these two factors are negative correlation. Fourth, silenced RAGE can obstruct this effect of AGEs.

The exact origin and phenotype of progenitor cells that differentiate into new endothelium and other vascular cells are still a matter of intensive research [21] and the relative contributions of progenitor-cell-mediated paracrine activation to angiogenesis or differentiation-dependent vasculogenesis to vascular adaptation are still a matter of controversial debate. DiI-acLDL/FITC-UEA-I double-positive MNCs include a heterogeneous group of progenitor-like cells with unknown potency for true vasculogenesis or for paracrine activation of angiogenesis. We have also found that most of the DiI-acLDL/FITC-UEA-I double-positive cells can be characterized by expression of the rat stem cell marker CD133 and the endothelial cell lineage antigen VEGFR-2. So, DiI-acLDL/FITC-UEA-I double-positive MNCs were presumed to be EPCs.

AGEs have been reported to induce cellular activation associated with chemotaxis, oxidative stress, cell proliferation, or programmed cell death. In our study, we found that AGEs, in a dose-dependent fashion, caused the generation of ROS in EPCs. Data also indicate that EPCs apoptosis increased with increasing AGEs concentrations, and the effect was attenuated during RAGE blocked. Significances of differences are as indicated: \( n=5, ^* P<0.01 \) group comparison, \( ** P<0.01 \) versus AGEs 400.
mRNA expression levels and activity of the anti-oxidative enzymes SOD and GSH-PX: expression levels and activity were reduced, so ROS levels should be increased. Lacking of the anti-oxidant enzyme can accelerate atherosclerosis [26]. And blocked RAGE protein expression by siRNA can obstruct this effect. In addition to specific effects of AGEs on vasculogenic function of EPCs cells in vitro, we have demonstrated a dose-dependent effect of AGEs on some biofunctions of EPCs. This study shows that AGEs can alter migration, adhesion, and proliferation of EPCs in a dose-dependent manner, and these effects were attenuated during blocking RAGE by siRNA. EPCs can localize to sites of neovascularization and participate in re-endothelialization after vascular injury, and differentiate into mature endothelial cells in situ. The number and functions of EPCs inversely correlate with the number of cardiovascular risk factors [27]. Also, disrupted migration, adhesion, and proliferation of EPCs may prevent re-endothelialization and even promote the process of atherosclerosis [28]. The effects of AGEs on EPCs functions may lead induce ROS formation and anti-oxidase decreasing in EPCs. Increased levels of ROS formation and decreased levels of anti-oxidases subsequently damage cellular structures and disrupt cellular functions, and EPCs apoptosis is increased.

Our data demonstrate that AGEs increase ROS generation, inhibit functions, alter anti-oxidant defenses, and activate apoptosis in EPCs. Thus, AGEs must reduce the vasculogenic potential of EPCs, which may be a new and important pathophysiological link to atherosclerosis. Our observations indicate that low concentration of AGEs in a patient with atherosclerosis may be necessary for successful progenitor cell therapy. Further investigation of the relationship between AGEs and EPCs will be required.

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