Advanced glycation end products impair the migration, adhesion and secretion potentials of late endothelial progenitor cells

Hong Li†, Xiaoyun Zhang†, Xiumei Guan, Xiaodong Cui, Yuliang Wang, Hairong Chu and Min Cheng*

Abstract

Background: Endothelial progenitor cells (EPCs), especially late EPCs, play a critical role in endothelial maintenance and repair, and postnatal vasculogenesis. Advanced glycation end products (AGEs) have been shown to impair EPC functions, such as proliferation, migration and adhesion. However, their role in the regulation of the production of vasoactive substances in late EPCs is less well defined.

Methods: Passages of 3–5 EPCs, namely late EPCs, were cultured with different concentrations (0–500 μg/ml) of AGEs, and the apoptosis, adhesion and migration were subsequently determined. The release of vasoactive substances, such as stromal cell-derived factor-1 (SDF-1), nitric oxide (NO), prostaglandin I2 (PGI2), plasminogen activator inhibitor-1 (PAI-1), tissue plasminogen activator (tPA), and in addition the activity of superoxide dismutase (SOD), were evaluated by ELISA. At the same time, the gene and protein expressions of CXCR4 were assayed by real-time RT-PCR and western-blot.

Results: AGEs promoted late EPC apoptosis. Moreover, AGEs impaired late EPC migration and adhesion in a concentration-dependent manner. Accordingly, the production of SDF-1 was decreased by AGEs. Although the CXCR4 expressions of late EPCs were up-regulated for AGE concentrations of 50, 100 or 200 μg/ml, a marked decrease was observed for the higher concentration of 500 μg/ml. Furthermore, co-culturing with AGEs decreased the levels of NO, t-PA, PGI2, and the activity of SOD but up-regulated the production of PAI-1.

Conclusion: Our data provide evidence that AGEs play an important role in impairing late EPC functions, which could contribute to the development of vascular diseases in diabetes.

Keywords: Endothelial progenitor cells, AGEs, Diabetes, Vasoactive substances
via non-enzymatic glycation, accumulate with age and abundantly increase in case of diabetes. They have been shown to be deposited in atherosclerotic lesion, and to promote diabetes-accelerated atherosclerosis [13,14]. Moreover, AGEs impair cell–matrix interactions and growth factor depletion [15]. These evidences suggest that the increased formation of AGEs contributes, at least in part, to the vascular damage in patients with diabetes. Recent studies have shown that AGEs promote EPC apoptosis, inhibit EPC proliferation, and impair EPC functions such as migration, adhesion and tube-forming ability [16,17]. However, growing evidence suggest that EPCs can keep endothelial integrity not only by differentiating into mature vascular endothelial cells, but also by secreting the soluble factors including a number of enzymes like matrix protein, growth factors and cytokines [18]. Although it would be interesting to examine the effects of AGEs on the secretion actions of EPCs, only limited studies have been conducted thus far. The present study therefore aims to evaluate the role of AGEs in regulating the release of vasoactive substances in late EPCs, such as NO, PGI2, PAI-1, tPA, together with the activity of SOD. At the same time, we also want to further confirm the effects of AGEs on the apoptosis, migration and adhesion of late EPCs, and to investigate the cellular basis, in particular the role of the SDF-1/CXC4 system.

Methods
Bone marrow mononuclear cell isolation and culture
Whole bone marrow was isolated from both the femurs and tibias of Sprague–Dawley rat (150 to 175 g) (Weifang medical College, China). The bone marrow mononuclear cells (MNCs) were fractionated by density gradient centrifugation (Histopaque®, 1083, Sigma, USA). MNCs were plated on dishes precoated with 5% fibronectin (Roche, Germany), and were maintained in complete EGM-2 medium (supplemented with EGM-2 bullet kit, including 5% fetal calf serum, recombinant rat VEGF, recombinant human bFGF, Invitrogen, USA). After 4 days in culture, unattached cells were removed by washing with PBS, after which fresh medium was added. Endothelial colonies subsequently appeared (on average 1 colony/10^7 or 10^8 plated mononuclear cells). Highly proliferative endothelial cells grew out from these colonies which then formed a confluent monolayer. Cells under passage 3–5, namely late EPCs, were used for the present study [19]. All animal protocols were approved by the local ethics committee at Weifang Medical College.

EPC identification
After 7 days of culture, EPCs were characterized by the uptake of 1,1′-dioctadecyl-3,3,3′,3′- tetramethylindocarbocyanine–labeled acetylated low density lipoprotein (Dil-acLDL, Molecular probes, USA), and by fluorescein isothiocyanate labeled Ulex europeaus agglutinin (FITC-UEA-1, Sigma, USA) staining. In short, the adherent cells were first incubated with Dil-acLDL (2 μg/ml) for 1 h, after which they were fixed in 2% paraformaldehyde for 10 min, and then stained with FITC-UEA-1 (10 μg/ml) for 1 h. After the staining, the samples were viewed with inverted fluorescence microscope (Leica, Germany). The early EPCs were moreover confirmed by the expressions of CD133 (eBioscience, USA, dilution 1:50) and Sca-1 (Becton Dickinson, USA, dilution 1:50). Cells under passage 3–5, namely late EPCs, were analyzed by FACS (Becton Dickinson, USA) for the expressions of vWF (Sigma, USA, dilution 1:200), VEGFR2 (eBioscience, USA, dilution 1:100), VE-cadherin (Becton Dickinson, USA, dilution 1:400) and PECAM-1(eBioscience, USA, dilution 1:100). The capability of EPCs to form capillary-like tubes in vitro was assessed on Matrigel.

FACS analysis of apoptosis
Late EPCs were treated with AGEs (Biovision, USA) for 24 h. The range of AGE concentrations used in all experiments was at a dose of 0–500 μg/ml which is representative of the plasma concentrations of AGEs found in diabetic patients [20]. Approximately 1 × 10^6 cells were double-stained with Annexin V–FITC and propidium iodide (PI) by using apoptosis detection kit (Becton Dickinson, USA) according to the manufacturer's instructions. Apoptotic cells (Annexin V+/PI-) were detected by FACS. The apoptotic percentage analysis was performed using Cell-Quest software (Becton Dickinson, USA).

EPC migration assay
The migratory function of EPCs was evaluated by a modified Boyden chamber (Costar, Cambridge, MA, USA) assay. Briefly, after incubation with serum-free medium for 6 h, EPCs were isolated with trypsin/EDTA, and then incubated with serum-free medium containing AGEs. A total of 4 × 10^5 EPCs were placed in the upper chamber while medium with VEGF (50 ng/ml) was placed in the lower chamber. The assays were conducted over a 24 h incubation period at 37°C in an incubator equilibrated with 5% CO2. The membrane was then washed gently with PBS, and fixed with 4% paraformaldehyde. Non-migrating cells were gently removed with cotton balls from the upper side of the membrane which was then stained by using 4′,6-diamidino-2-phenylindole (DAPI). The migration of EPCs was evaluated by counting the migrated cells in six random high-power (100×) microscope fields/well.

EPC adhesion assay
EPC adhesion assay were performed as previously described [21]. After being treated with different concentrations of AGEs for 24 h, EPCs were washed with PBS, and then gently detached with 0.25% trypsin/ EDTA. After centrifugation
and resuspension with serum-free medium, equal cell numbers were seeded on fibronectin coated culture dishes, and incubated for 30 min at 37°C. Adherent cells were counted independently in six random high-power (×100) microscope fields (HPF)/well by three observers unaware of the treatments.

**Assay of the expressions of CXCR4 of EPCs by real-time RT-PCR and western blot**

Total cellular RNA was isolated with TRIzol reagent (Invitrogen, USA) and reverse-transcribed to cDNA using the SYBR® ™ PrimeScript™ RT-PCR Kit (Takara, Japan) at 37°C for 15 min. Gene expressions were evaluated by SYBR® Premix Ex Taq™ (Takara, Japan). Rat CXCR4 was amplified with the sense primer 5′-AGC AGG TAG CAG TGA CCC TCT GA-3′ and the anti-sense primer: 5′-GAA GCA GGG TTC CTT GTT GGA GT-3′. GAPDH (sense primer: 5′-GCC ACA GTC AAG GCT GAG AAT G-3′, anti-sense primer: 5′-ATG GTG GTG AAG ACG CCA GTA-3′) was used as a housekeeping gene, in order to normalize the expression target gene. All primers were used at a final concentration of 0.4 μmol/L. The thermal cycling conditions were as follows: 30 seconds at 95°C for pre-denaturation, 40 cycles for 15 seconds at 95°C for denaturation, 1 minute at 59°C for annealing, and 10 seconds at 72°C for elongation. At the end of each cycle, the fluorescence emitted by the SYBR Green I was measured. After completion of the cycling process, samples were immediately subjected to a temperature ramp for melting curve completion of the cycling process, samples were immediately subjected to a temperature ramp for melting curve completion of the cycling process. The membranes were blocked in 5% milk-TBST, followed by overnight incubation with the primary antibodies against CXCR4. The membranes were then washed with TBST, and incubated with secondary antibody conjugated to HRP (Santa Cruz, USA, dilution 1:1000). Immunoreactive bands were visualized by chemiluminescence (ECL Amersham Pharmacia).

**Assay of secretion actions of EPCs**

To measure the secretion actions of EPCs, the cultures were washed and re-fed with equal amounts of serum-free medium containing different concentrations of AGEs for 24 h. The medium was collected and 10 × concentrated by centrifugation for 20 min at 5000 × g at 4°C using Ultrafree-4 centrifugal filter tubes with Biomax-5 membrane (Millipore, USA). The levels of SDF-1, NO, t-PA, PAI-1, PGI2, and the activity of SOD in the cell culture supernatants were measured by sandwich enzyme-linked immunosorbent assay (ELISA, RD, USA) according to the manufacturer’s instructions. Briefly, standard or testing sample (50 μl) was added to the 96-well plate containing immobilized monoclonal antibodies against a kind of factor. After mixing by shaking gently, the plate was incubated at 37°C for 30 min. The wells were then washed five times, and incubated with HRP-conjugate reagent at 37°C for 30 min. Afterwards, chromogen solution A and chromogen solution B were added to each well. The reaction was terminated after 20 min by adding the stop solution. Blank well was taken as zero, and the optical density (OD) was measured with an enzyme-linked immunoabsorbent assay reader (Bio-Rad Laboratories, USA).

**Statistical analyses**

Unless otherwise indicated, results are reported as mean ± SE from at least 4 independent experiments. Statistical analyses were performed by one-way ANOVA, followed by Tukey-Kramer post hoc test for multiple comparisons, and p < 0.05 was considered to be statistically significant. All data were analyzed using SPSS software (version 15.0; SPSS, Chicago, IL, USA).

**Results**

**Characterization of bone marrow-derived late EPCs**

The bone marrow-derived MNCs that initially seeded were round (Figure 1A). The distinct colonies formed after 48 h, reaching a peak on the seventh day. The colonies of early EPCs appeared with the round cells in the centers and the typical spindle cells at the peripheries after 7 days (Figure 1B), and were identified as double-positive for Dil-acLDL uptake and lectin binding affinity (Figure 1D, E, F). Further FACS analysis revealed that the respective expressions of CD133 and Sca-1 were around 24.5% and 3.3% in early EPCs (Figure 1G). After 3–4 weeks, the 3–5 passage cells (late EPCs) showed characteristic homogeneity and cobblestone-like morphology similar to mature endothelial cells as previously reported (Figure 1H) [23]. Moreover, the majority of the cells expressed endothelial-specific markers: vWF, VEGFR-2, VE-cadherin and PECAM-1 (Figure 1H) [10]. The angiogenic property of the EPCs was also studied. As shown in Figure 11 and Figure 1J, late, but not early, EPCs successfully formed tubulike structures on Matrigel.

**Effects of AGES on late EPC apoptosis**

Late EPCs were incubated with different concentrations of AGEs (0, 50, 100, 200 and 500 μg/ml) for 24 h. As shown in Figure 2, AGEs induced apoptosis in late EPCs.
The number of apoptotic cells (Annexin V+/PI-) was observed to increase with higher concentrations of AGEs, reaching a peak at 100 μg/ml, and subsequently saturating to a fixed value.

Effects of AGEs on late EPC migration, adhesion and SDF-1/CXCR4 system

To examine the effects of AGEs on late EPC migration, cells were treated for 24 h with different concentrations of AGEs in the upper compartment of a Boyden chamber, the lower compartment of which contained EBM-2 medium with VEGF. The AGEs were observed to inhibit late EPC migration in a concentration-dependent manner, with a 4-fold decrease at the maximal concentration tested (500 μg/ml, Figure 3A). Moreover, late EPC adhesion on FN was also significantly reduced in a concentration-dependent manner with adherent cells at less than 38% of the control value when applied to the maximal dose of AGEs (500 μg/ml, Figure 3B). To determine whether the impaired migration and adhesion observed in AGE-stressed EPCs was related to the SDF-1/CXCR4 system, the release of SDF-1 was measured by ELISA. At the same
time, the mRNA and protein expressions of CXCR4 were assessed by real-time RT-PCR and western blot, respectively. The AGES were found to induce a decrease in the SDF-1 secretion in a dose-dependent manner (Figure 3C). However, EPCs treated with 50, 100 and 200 μg/ml of AGES displayed higher mRNA and protein expressions of CXCR4 than those in the untreated sample. Interestingly, 500 μg/ml of AGES markedly decreased the CXCR4 mRNA and protein levels of late EPCs (Figure 3D, E).

**Effects of AGES on the productions of the vasodilators, NO and PGI2, in late EPCs**

To investigate the effects of AGES on the productions of the vasodilators in late EPCs, cells were incubated for 24 h in medium containing different concentrations of AGES (0 ~ 500 μg/ml). The cultured media were collected, and the amounts of NO and PGI2 released from the late EPCs were determined by ELISA. Given that PGI2 has a short half-life (<20 minutes at physiological pH), levels of 6-keto-prostaglandin F1α (6-keto-PGF1α), the PGI2 stable degenerative product, were quantified to evaluate the PGI2 secretion from late EPCs. The results show that the incubation of late EPCs with higher concentrations of AGES (200 and 500 μg/ml) significantly decreased the production of NO, compared with that in the control medium. For the lower concentrations of AGES (50 and 100 μg/ml), however, no such decrease was observed (Figure 4A). The production of 6-keto-PGF1α by AGE-stressed EPCs was reduced in a dose-dependent manner. (Figure 4B)

**Role of AGES on the activity of SOD in late EPCs**

The bioactivity of SOD was measured in late EPCs. As shown in Figure 5, incubation of cells with higher concentrations of AGES decreased the SOD activity in EPCs as compared to the control medium. The SOD activity of EPCs was reduced by 25.9% and 43.7% in AGE concentrations of 200 and 500 μg/ml, respectively.

**Influence of AGES on PAI-1 and t-PA secretions from late EPCs**

Since the balance between PAI-1 and tPA determines the fibrinolytic activity, we also investigated the effects of...
AGEs on the productions of PAI-1 and tPA from late EPCs. To this end, late EPCs were exposed to AGEs at concentrations of 0~500 μg/ml for 24 h, after which they were tested for PAI-1 and tPA productions by ELISA. Incubation of late EPCs with 500 μg/ml of AGEs led to a significant decrease in the production of t-PA. However, no statistically significant decrease was observed for low concentrations of AGEs (Figure 6A). In contrast, incubation of late EPCs with 500 μg/ml AGEs led to an increase in the production of PAI-1 (Figure 6B).

Discussion

AGEs, as a result of chronic hyperglycemia in diabetic patients, have been identified as one of the key risk factors in diabetic-accelerated atherosclerosis [24]. Cell activation, proinflammatory cytokine release and oxidative stress, induced by the interaction of AGEs with their receptors, may lead to endothelial injury and dysfunction [25,26]. Growing evidence suggests that EPCs, mobilized from bone marrow and other sites, contribute to the structural integrity of the vasculature, and also restore endothelial dysfunction [3]. Recently, Palombo et al [27] have reported that a significant reduction in the number of EPCs is related to carotid intima-media thickness, an induction of early atherosclerosis in diabetes mellitus. Moreover, the frequency of EPCs in DM patients is negatively correlated with the levels of hemoglobin A1c (HbA1c) [28], and DM patients with satisfactory glycemic control (defined by Hemoglobin A1c, HbA1c < 6.5%) have been found to have significantly higher circulating EPC counts [29]. On the contrary, reduction in circulating and soluble form of AGEs by azelnidipine [30] may have beneficial effects on glucose tolerance, insulin sensitivity, the inflammatory state, and number of EPCs [31].

Sun et al [32] have reported that the receptors of AGEs (RAGE) are expressed on EPCs at a low level in normal conditions, and that AGEs significantly increase the RAGE expression, and promote EPC apoptosis in a dose-dependent manner (50~300 μg/ml). In agreement with those findings, we have here shown that AGEs...
induce apoptosis in late EPCs, with the apoptosis rate, however, reaching peak at a concentration of 100 μg/ml, after which it settles at a constant value even when the AGE concentration is increased further. One possible explanation for the differences between the observed relationship between apoptosis rate and AGE concentration could be the different types of EPCs used in these studies. Late EPCs were used in our study, while Sun et al. used early EPCs in their experiments.

Previous investigations have also demonstrated that AGEs impair EPC functions, such as migration, adhesion and tube formation [16,17]. Our results further confirm that AGEs inhibit migration and adhesion in late EPCs. It is then natural to ask what the mechanisms behind this might be. SDF-1 and its transmembrane receptor CXCR4 play a pivotal role in regulating the trafficking of immature stem cells [33,34]. Enhanced SDF-1 expression and production is essential for the migration and adhesion of stem cells [35]. It has been shown that ex vivo exposure of EPCs to SDF-1 induces cell migration and adhesion [36]. Moreover, Smadja et al. [37] have demonstrated that the angiogenic ability of EPCs may be mediated by an autocrine mechanism involving SDF-1/CXCR4. To characterize the role of the SDF-1/CXCR4 system in the AGE-induced decrease of the migration and adhesion of late EPCs, we quantified the expressions of SDF-1 and CXCR4. We have observed here that AGEs induce a reduction in SDF-1. Intuitively, one could then expect that AGEs might result in a decrease in CXCR4 as well. In our study, however, we found that AGEs led to the CXCR4 overexpression on the late EPCs, with the overexpression increasing as a function of the AGE concentration only up to a certain point (200 μg/ml), whereas beyond this, at a very high concentration (500 μg/ml), the CXCR4 expression levels were seen to decline. Thus the elevated CXCR4 expression may account for a compensatory response to the defective SDF-1 in the AGE-stressed EPCs while a high concentration of AGEs can destroy the intrinsic compensatory ability of late EPCs.

Due to the fact that the release of vasoactive factors by EPCs also contributes to the vasculature integrity and homeostasis [18], we explored in addition the influence of AGEs on late EPC secretion functions. The results show that AGEs cause a reduction in NO, PGI2 and tPA. Furthermore, AGEs were identified to decrease the SOD activity of EPCs. At high concentrations, however, the AGEs were seen to increase the secretion of PAI-1 by EPCs.

NO is a major vasodilator and a key survival factor of the endothelium. The reduction in NO production is possibly contributing to the development of endothelial dysfunction and atherosclerosis in diabetes mellitus [38]. Ozuyaman and colleagues have demonstrated that NO
can stimulate EPC mobilization from bone marrow stem cell niches to the peripheral circulation [39]. Moreover, it has also been shown that the treatment of EPCs from diabetes patients with an NO donor drug normalizes their migration [40], and that SDF-1α restores EPC homing to wounds in diabetic mice through an NO-dependent mechanism [41]. Urao et al have reported that transplantation of autologous EPCs overexpressing eNOS in injured vessels enhances the vasculoprotective properties of reconstituted endothelium [42]. Therefore, NO which is produced by the EPCs themselves may create a favorable and optimal environment in regulating their functions. Liang et al [21] reported that the expression of phosphorylated-eNOS is reduced by AGEs in EPCs. In line with this finding, our results show that a 24 h incubation with AGEs significantly decreases bioavailable NO of late EPCs, which would be one of the determinants of vascular damage in diabetic patients.

In addition to NO, PGI2 is not only a potent vasodilator contributing to the protection and maintenance of homeostasis in vasculature but also an inhibitor of platelet activation. It has been shown that PGI2 has a direct effect on EPC functions in an autocrine or paracrine manner through an NO-dependent mechanism, and EPCs from prostacyclin receptor (IP−/−) mice fail to promote re-endothelialization and vessel repair [43]. In the present study, our results show that a 24 h incubation with AGEs significantly decreases bioavailable NO of late EPCs, which would be one of the determinants of vascular damage in diabetic patients.

It has become evident that the interaction between NO and O2 is important in the development of endothelial dysfunction [46]. EPCs have high intracellular expression levels of SOD, which can protect them against oxidative stress [47]. Thum et al have reported that increased NAD (P)H oxidase activity results in increased O2 generation and reduced NO bioavailability because O2 inactivates NO and uncouples eNOS in EPCs [48]. Moreover, the increased O2 is closely associated with the reduced extracellular SOD activity [49]. Thus the addition of SOD may scavenge O2, increase NO bioavailability and/or prevent the uncoupling of eNOS in glucose-stressed EPCs [50]. We have here shown that AGEs dose-dependently inhibited SOD activity, which may account for AGEs decreasing bioavailable NO of late EPCs. Thus, AGEs may increase the risk of developing cardiovascular diseases by decreasing the SOD activity of EPCs.

Clinical and experimental studies suggest that thrombus formation and plaque rupture are other major aspects of cardiovascular diseases [51]. t-PA is one of the most important fibrinolytic substances which regulates fibrinolytic activity and prevents thrombus formation. Serving as the primary inhibitor of t-PA, PAI-1 plays a critical role in the regulation of fibrinolysis. The balance between these proteins is known to control the development of thrombosis. A recent study has shown that EPCs can also produce t-PA, and that the amount secreted by EPCs is comparable to that secreted by mature endothelial cells [52]. Thus we have here also studied the effects of AGEs on the secretion of t-PA and PAI-1 from late EPCs. We have shown that incubation of late EPCs with high concentrations of AGEs leads to a significant decrease in the production of t-PA, while increasing the production of PAI-1. The different effects of AGEs on t-PA and PAI-1 might lead to an imbalance between them, inducing EPCs to become procoagulative and hypofibrinolytic.

Conclusion
Taken together, the present study demonstrates that AGEs exert deleterious effects on EPC functions, such as
migration, adhesion and secretion action. It provides evidence that diabetes via AGEs may contribute to the functional defects of the endothelium in pathological situations by causing the dysfunction of EPCs. Furthermore, these findings give further insight into the complex cellular mechanisms of EPCs for impaired vascular repair and neovascularization in diabetic patients.

Abbreviations
EPCs: Endothelial progenitor cells; AGEs: Advanced glycation end products; SDF-1: Stromal cell-derived factor-1; NO: Nitric oxide; PGI2: Prostaglandin I2; vWF: Von Willebrand factor; VEGFR2: Vascular endothelial growth factor receptor 2; VE-cadherin: Vascular endothelial cadherin; PECAM-1: Platelet endothelial cell adhesion molecule-1.

Competing interests
The authors declare that they have no competing interests.

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Authors’ contributions
HL, XYZ and MC were the principal investigators, involved in designing the study and writing the manuscript. XMG coordinated the study and performed statistical analyses. XDC, YLW and HRC were involved in cell culture, western blot and writing parts of the manuscript. All authors participated in writing the final version of the manuscript.

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