Research Article

AGEs/RAGE Promote Osteogenic Differentiation in Rat Bone Marrow-Derived Endothelial Progenitor Cells via MAPK Signaling

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Systemic vascular impairment is the most common complication of diabetes. Advanced glycation end products (AGEs) can exacerbate diabetes-related vascular damage by affecting the intima and media through a variety of mechanisms. In the study, we demonstrated that AGEs and their membrane receptor RAGE could induce the differentiation of EPCs into osteoblasts under certain circumstances, thereby promoting accelerated atherosclerosis. Differentiation into osteoblasts was confirmed by positive staining for Dil-acetylated fluorescently labeled low-density lipoprotein and FITC-conjugated Ulex europaeus agglutinin. During differentiation, expression of receptor for AGE (RAGE) was significantly upregulated. This upregulation was attenuated by transfection with RAGE-targeting small interfering (si)RNA. siRNA-mediated knockdown of RAGE expression significantly inhibited the upregulation of AGE-induced calcification-related proteins, such as runt-related transcription factor 2 (RUNX2) and osteoprotegerin (OPG). Additional experiments showed that AGE induction of EPCs significantly induced ERK, p38MAPK, and JNK activation. The AGE-induced upregulation of osteoblast proteins (RUNX2 and OPG) was suppressed by treatment with a p38MAPK inhibitor (SB203580) or JNK inhibitor (SP600125), but not by treatment with an ERK inhibitor (PD98059), which indicated that AGE-induced osteoblast differentiation from EPCs may be mediated by p38MAPK and JNK signaling, but not by ERK signaling. These data suggested that AGES may bind to RAGE on the EPC membrane to trigger differentiation into osteoblasts. The underlying mechanism appears to involve the p38MAPK and JNK1/2 pathways, but not the ERK1/2 pathway.
1. Introduction

Diabetes is one of the most common chronic diseases in the world. In 2019, 8.8% of the adult population (463 million people) was reported to have diabetes [1]. Systemic vascular impairment is the most common complication of diabetes and is one of the leading causes of mortality in diabetic patients. Approximately 4.2 million people died from diabetes in 2019 [1]. Protection of patients with diabetes against vascular injury has therefore attracted significant attention from researchers [2]. The incidence of vascular complications was significantly increased in patients with diabetes compared with those without diabetes [3, 4]. Advanced glycation end products (AGEs) are excess sugars and protein-bound products closely associated with vascular calcification. AGEs are reported to significantly promote the development of atherosclerosis through signaling mediated by receptor for AGE (RAGE) [5]. Vascular calcification is one of the pathological features of atherosclerosis. Atherosclerotic lesions are characterized by their origin in the intima of the vessel, accompanied by accumulation of lipids and complex carbohydrates, hemorrhage, and thrombosis, and subsequent simultaneous involvement of the media. When the disease progresses to the arterial lumen being blocked, it will directly lead to ischemia or even necrosis of the tissues or organs supplied by the artery [6]. Current views suggest that vascular calcification is not a simple process of passive degeneration, but, rather, an active process initiated by a variety of cells. It is similar to bone development and involves active bone formation, including the development of osteoblasts and the expression of active markers on the bone surface [7]. Furthermore, AGEs have been shown to induce the differentiation of human periodontal ligament stem cells into osteoblasts [8], indicating that they may have potential to promote the differentiation of EPCs into osteoblasts during the process of diabetes-related vascular injury.

Asahara et al. [9] first discovered that human peripheral blood CD34+ hematopoietic stem cells can differentiate into mature endothelial cells. The differentiation of CD34+ hematopoietic stem cells into mature endothelial cells promotes angiogenesis in ischemic tissue; this hematopoietic stem cell type was thereafter known as endothelial progenitor cells (EPCs) [9]. Since then, researchers have performed the "double-swallow" test with FITC-UEA-1 and Dil-ac-LDL to successfully identify EPCs [10]. Blood vessel injury triggers signaling that recruits EPCs to the site of vascular injury where they differentiate into mature endothelial cells that participate in vascular repair [11]. Endothelium calcification is associated with vascular repair disorders caused by abnormal lipid metabolism, inflammation, and/or EPC dysfunction. A previous study has shown that EPCs can promote the repair of damaged blood vessels, thereby slowing the progression of atherosclerosis [12]. However, clinically, the therapeutic effects of EPCs are far less impressive than their original proponents expected, particularly in diabetic patients. EPCs can simultaneously express surface markers associated with stem cells as well as those associated with endothelial cells [13, 14]. It was therefore hypothesized that EPCs still have multidirectional differentiation potential. In certain circumstances, EPCs with multidirectional differentiation potential may differentiate into osteoblasts, thereby slowing the repair of vascular damage and promoting atherosclerosis [15].

The MAPK pathway is a crucial signaling pathway that is involved in a variety of physiological cellular responses, including cell growth, development, division, apoptosis, and functional synchronization [16]. Notably, MAPK serves a role in the differentiation of osteogenic mesenchymal stem cells [17], as well as the differentiation of 3T3-L1 preadipocytes [18]. Whether AGEs can promote osteogenic differentiation of EPCs and the specific mechanism has not been reported. The aim of the present study was to explore whether AGEs could trigger the differentiation of EPCs into osteoblasts through activation of the MAPK pathway. The circumstances under which AGEs induced the differentiation of EPCs into osteoblasts through activation of the MAPK pathway were also assessed. EPCs were successfully differentiated into osteoblasts by exposing them to AGEs, and the role of the MAPK signaling pathway during this differentiation was determined.

2. Materials and Methods

2.1. Isolation of EPCs. Twenty male Sprague-Dawley rats (weight, 120-150 g; age, 6-8 weeks, 4 rats per cage) were provided by the Animal Laboratory Center of Southwest Medical University. The rats were fed with free access to food/water in a 20-25°C and 55 ± 5% humidity room with light/dark cycling every 12 h. The experiments performed in the present study were reviewed and approved by the Ethics Committee of Southwest Medical University (No. 2015103051). In the course of the experiment, the rats were fed and handled in strict accordance with Chinese Regulations on the Administration of Laboratory Animals (revised in 2014) (http://www.gov.cn/gongbao/content/2014/content_2692743.htm) to ensure the welfare of the animals. Rats were sacrificed by cervical dislocation at the end of the study period. The femurs and tibias of the rats were removed and punched with a sterile needle to create holes on each end of the femurs obtained. The bone marrow was washed out with PBS, centrifuged for 5 min at 400 × g at room temperature, and then mixed by pipetting with 5 ml PBS. The cell suspension obtained was layered over Histopaque 1077 (Sigma-Aldrich; Merck KGaA) and centrifuged for an additional 30 min at 400 × g at room temperature. The cellular layer interface was collected and washed three times with PBS. The mononuclear cells collected were seeded on T-75 tissue culture flasks at a density of 1.25 × 10^7 cells/ml with EGM-2MV medium (Lonza Group Ltd.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Waltham, MA, USA), 1% penicillin (10,000 U/ml)-streptomycin (10,000 μg/ml), and EGM-2MV SingleQuots (Lonza Group Ltd.) (together termed complete EGM-2MV medium) in a humidified incubator at 37°C and 5% CO2. After incubation for 48 h, nonadherent cells and debris were aspirated, and adherent cells were cultured with fresh complete EGM-2MV medium daily for 7 days (media were exchanged every 48 h).
2.2. Identification of EPCs. Adherent cells were incubated at 37°C and 5% CO₂ with 1,10-dioctadecyl-3,3,30,30-tetramethyl-indocarbocyanine-labeled acLDL (DiI-acLDL) (Molecular Probes; Thermo Fisher Scientific) for 4 h, fixed in 2% paraformaldehyde for 20 min at room temperature, and then counterstained with FITC-labeled lectin from Ulex europaeus agglutinin (UEA-1) (Sigma-Aldrich; Merck KGaA). Fluorescent images were captured using a laser-scanning confocal microscope.

2.3. EPC Treatment. EPCs were divided into 4 groups: (i) control group incubated with complete EGM-2MV medium only; (ii) a group incubated with complete EGM-2MV medium+40 mg/l AGEs (Biovision, Japan); (iii) a group incubated with complete EGM-2MV medium+10 mmol/l β-glycerophosphate; and (iv) a group incubated with complete EGM-2MV medium+40 mg/l AGEs+10 mmol/l β-glycerophosphate.

To investigate the role of RAGEs, the PECs were divided into four groups: (i) control (PBS treatment); (ii) 100 mg/l AGEs; (iii) pHBLV-U6-Scramble-ZsGreen-Puro transfection (SnapGene; multiplicity of infection (MOI) 50/each)+100 mg/l AGEs; and (iv) small interfering (si)RNA against RAGE (siRAGE; MOI 50/each)+100 mg/l AGEs.

2.4. Identification of Osteoblasts. The EPCs on 24-well tissue culture plates were induced with 10 mmol/l β-glycerophosphate+40 mg/l AGEs for 7 days. Differentiated osteoblasts were identified by examining immunofluorescence staining. Briefly, the cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. For intracellular staining, cells were permeabilized with 0.1% Triton X-100 (Beyotime Biotechnology, Shanghai, China) for 15 min, washed by PBS (5 min, three times) at room temperature, reacted with osteocalcin (OCN), CD34, CD133, VE-cadherin, or runt-related transcription factor 2 (RUNX2) antibodies (Abcam Cambridge, United Kingdom) overnight at 4°C, and, when needed, reacted with secondary antibodies at room temperature for 2 h. Nuclei were stained with DAPI. The results were observed under a CKX53 confocal microscope (OLYMPUS, Tokyo, Japan).
2.5. Western Blotting. Collected cells were lysed with protein extract buffer (1 ml Protein Extract Buffer (Beyotime Biotechnology, Shanghai, China) with a 5:1 mixture of protease inhibitors, 5 μl PMSF, and 5 μl phosphatases) at 4°C by sonication with 40 Hz for 10 seconds each, with 5 min intervals on ice. The supernatant was collected as protein samples after centrifugation at 14,000 × g for 30 min at 4°C. Each 20 μg protein sample was separated by 10% SDS-PAGE and then transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk and incubated for 12 h at 4°C with the following primary antibodies (1:1,000; all purchased from Abcam): goat anti-rabbit osteoprotegerin (OPG) IgG, RUNX2 IgG, BMP-2 IgG, RAGE, and GAPDH. Membranes were then washed with Tris-buffered saline containing 0.2% Tween-20 (TBS-T), incubated with horseradish peroxidase-conjugated anti-goat IgG antibody (1:5,000) secondary antibody for 1 h, and then washed with TBS-T buffer three times. The signals were visualized using Enhanced Chemiluminescence Detection Reagent (Engreen, Beijing, China). GAPDH was used as the loading control. Target protein expression was normalized to the levels of the respective GAPDH bands using Quantity One software (Version 4.6.2).

2.6. siRAGE and Lentiviral Transduction. siRNA (5'-GGAGTGCAACTGAACTTTGTTT-3') targeting rat RAGE (rat RAGE gene ID: NM_053336.2) was designed and synthesized by Hanbio Biotechnology (Shanghai, China). Empty vector (pHBLV-U6-Scramble-ZsGreen-Puro, 3 × 10⁸ TU/ml) (Hanbio Biotechnology, Shanghai, China) and the siRAGEs (MOI 10, 30, and 50, respectively) were transduced into EPCs using the ViraPower Lentiviral Expression System (Thermo Fisher Scientific) according to the manufacturer’s instructions to quantify transfection. Finally, the optimal MOI value was 50 (data not shown). Transfection efficiency was evaluated after 72 h using a fluorescence microscope (data not shown). Thus, 72 h transfection time was used for subsequent experiments. The optimal MOI value (MOI50) for the siRNA used was analyzed by western blotting as well.
2.7. MAPK Signaling Analysis. Serum-starved second-generation EPCs were treated with 100 mg/l AGEs and terminated at 0, 5, 15, or 30 min in a 37°C incubator. Protein samples were extracted as described for western blot analysis. Protein levels of phosphorylated ERK (CST, CA, USA), p38MAPK (Santa Cruz, CA, USA), and JNK (Biotechnology, CA, USA) were analyzed by western blotting. To analyze a specific pathway, the cells were pretreated with 20 mmol/l SB20358 (Selleck Chemicals, Texas, USA) to inhibit p38MAPK, with 50 mmol/l PD98059 (Selleck Chemicals) to inhibit ERK, or with 50 mmol/l SP600125 (Selleck Chemicals) to inhibit JNK inhibitor for 1 h prior to treatment with AGEs. Equivalent volumes of DMSO were used for the control treatment.

2.8. Statistical Analysis. Data analysis was performed using SPSS version 20.0 (IBM Corp.). All data are presented as the mean ± the standard error of the mean and analyzed using a one-way ANOVA with Bonferroni post hoc comparisons. P < 0.05 was considered to indicate a statistically significant difference.

3. Results

3.1. Isolation of EPCs from Rat Bone Marrow. Mononuclear cells isolated from rat bone marrow were incubated in complete EGM-2 medium. On the 3rd day, a large number of cells appeared to exhibit a round morphology (Figure 1(a)). On the 7th day, colonies of spindle-shaped cells were observed.
**Figure 5:** siRAGE transduction reduces the AGE-induced increase in RAGE expression in EPCs and reduces the expression levels of calcification-related proteins in EPCs. (a) Representative western blotting images and densitometric analysis of RAGE protein expression in EPCs stimulated with the indicated concentration of AGE for 7 days. (c) RAGE protein expression levels were detected and semiquantified using western blotting in the EPCs induced by 100 μg/ml AGE for 7 days, after transfection with siRAGE, EV, or treatment with PBS as the control. (e) EPCs were transfected with siRAGE, empty vector (EV-AGEs), or control (EGM-2MV) then induced with 100 μg/ml AGE for 7 days. Cells not treated with AGEs were used as the controls. Expression levels of cellular calcification-related proteins OPG and RUNX2 were detected by western blotting. GAPDH was used as the loading control. Each data point represents the results of three independent assays. Comparison with control, ▲\( P < 0.05 \); AGEs 100 mg/ml compared with other groups; *\( P < 0.05 \); comparison with AGEs alone; *\( P < 0.05 \); comparison with EV-AGEs, *\( P < 0.05 \); AGE: advanced glycation end product; EPC: endothelial progenitor cell; EV: empty-vector–transfected control group; OPG: osteoprotegerin; RUNX2: runt-related transcription factor 2; siRAGE: small interfering RNA against receptor for AGE.
3.2. β-Glycerophosphate+AGE Induce the Differentiation of EPCs into Osteoblasts. EPCs in the intervention group (AGEs+B-gly) were treated with 10 mmol/l β-glycerophosphate+40 mg/l AGEs for 7 days. Phenotypic changes in the EPCs were detected by immunofluorescence staining. Compared with the control cells, the AGE-induced cells had notably decreased levels of the stem cell marker CD133 (Figure 2(a)) and the endothelial cell marker VE-cadherin (Figure 2(b)). The expression of calcification-related markers RUNX2 (Figure 2(c)) and OCN (Figure 2(d)) was analyzed. Compared with untreated cells, the coexpression of endothelial marker CD34 and osteogenic marker OCN in the same EPCs was significantly increased after culturing with AGEs+β-gly. (Figure 3). The results indicated that the AGEs+β-gly-induced EPCs lost some aspects of the endothelial cell phenotype and acquired the phenotype of osteoblasts (coexpression of CD34 and OCN).

The results of western blot analysis showed that, compared with the 10 mmol/l β-glycerophosphate group, the 40 mg/l AGEs+10 mmol/l β-glycerophosphate group had significantly higher expression levels of calcification-related proteins OPG, RUNX2, and BMP-2 (all \( P < 0.05 \); Figure 4). In addition, protein expression was higher in the β-glycerophosphate group than in the NC and AGE groups (Figure 4). The OPG expression level in the AGE group was increased compared with that in the NC group (Figure 4), even though no statistical significance was found. These data suggested that β-glycerophosphate successfully induced the differentiation of EPCs into osteoblasts and AGEs could facilitate this process.

3.3. AGE Induced Upregulation of RAGE. We explored the effect of various concentrations of AGEs on RAGE in the next investigations. RAGE expression was significantly increased in cells stimulated with 10-80 μg/ml AGE (\( P < 0.05 \)). Compared with the 10-80 μg/ml AGE treatment groups, 100 μg/ml AGE resulted in further upregulation of RAGE expression (\( P < 0.05 \))(Figure 5(a)).

3.4. siRNA-Mediated Knockdown of RAGE Expression in EPCs. The crucial role of RAGE in AGE-induced osteoblast differentiation was further investigated using siRNA targeting rat-specific RAGE mRNA. Western blotting showed that RAGE expression levels were knocked down significantly in the cells transduced with siRAGE compared with the untransfected control cells and empty-vector-transfected (EV) cells (\( P < 0.05 \); Figure 5(c)).

3.5. siRAGE Prevents AGE-Induced Differentiation of Osteoblasts from EPCs. siRAGE transfected EPCs were treated with 100 μg/ml AGEs to induce osteoblast differentiation. Transfection with siRAGE prevented AGE-induced differentiation of osteoblasts from EPCs, as the protein expression levels of calcification-related proteins (OPG and RUNX2) were significantly decreased in the siRAGE-treated cells compared with those in the AGEs and EV+AGE groups (Figure 5(e)). These results suggested that AGEs promote osteogenic differentiation of EPCs via RAGE.

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**Figure 6:** MAPK pathway phosphorylated protein expression for AGE-induced activation of ERK, p38MAPK, and JNK in EPCs. EPCs were stimulated with 100 μg/ml AGE for 0, 5, 15, or 30 min. (a) Phosphorylation levels of ERK, p38, and JNK were analyzed by western blotting. Total MAPK, p38, and JNK expression levels were used as the corresponding controls; GAPDH was used as a loading control. (b) Average phosphorylation levels of ERK, p38, and JNK were determined by presenting the ratio of phosphorylated/total protein expressions. Each data point represents the results of three independent repeats. \( * P < 0.05 \) vs. 0 min. AGE: advanced glycation end product; EPC: endothelial progenitor cell; p-: phosphorylated.
3.6. p38MAPK and JNK Pathways Mediate AGE-Induced Osteoblast Differentiation from EPCs.

Phosphorylation levels of ERK, p38MAPK, and JNK were further analyzed by western blotting of EPCs stimulated with 100 μg/ml AGEs for 0, 5, 15, or 30 min. Significant activation of these proteins was observed after 5 min of stimulation (all \( P < 0.05 \); Figure 6). The expression of phosphorylated protein decreased gradually over time. Pretreatment with 50 μmol/l PD98059, 50 μmol/l SB203580, or 20 μmol/l SP600125 blocked AGE-induced activation of ERK, p38, and JNK (all \( P < 0.05 \); Figure 7). The effects of the inhibitor treatments on AGE-induced osteoblast differentiation were investigated as well. The EPCs were pretreated with PD98059, SB203580, or SP600125 for 24 h; then, the medium was replaced to expose cells to AGE for 7 days, and the expression of osteoblast-related proteins was detected by western blotting. The results suggest that the upregulation of calcification-related proteins RUNX2 and OPG was significantly prevented by treatment with SB203580 and SP600125, but not PD98059 (Figure 8). These data suggested that the AGE-induced osteoblast differentiation from EPCs was mediated by p38 and JNK signaling, but not by ERK signaling.

4. Discussion

Since the discovery and description of EPCs, researchers have developed a keen interest in its ability to repair vascular. A variety of studies have demonstrated that EPCs can overcome endothelial dysfunction and reduce atherosclerosis risk [19, 20]. However, the use of stem cells to treat vascular diseases has been full of controversy. Studies have shown that in humans, cardiovascular risk factors, such as diabetes, hypertension, and smoking, impair number and function of EPCs, potentially restricting the therapeutic potential of progenitor cells [21]. The damage of EPCs seems to significantly promote the progression of diabetic atherosclerosis and atherosclerotic disease [22]. In our study, the differentiation of EPCs into osteoblasts may be one of the potential risks of exacerbation of atherosclerosis in patients with diabetes.

Numerous studies have suggested that vascular calcification is not a simple process of calcium deposition, but rather a highly regulated process, similar to bone development [23]. Increasing evidence indicates that there is a close relationship between bone metabolism and the vascular system.
EPCs are not only capable of differentiation into endothelial cells that repair damaged blood vessels but have also potential for multidirectional differentiation, occasionally to cell types that participate in bone metabolism [24]. The experiments performed in the present study showed that the levels of stem cell marker CD133 and endothelial cell marker VE-cadherin decreased after AGE and β-glycerophosphate induction, whereas the levels of calcification-related markers RUNX2 and OCN increased. These findings indicated that EPCs may transdifferentiate into osteoblasts. Studies by Gössl et al. [15, 25] showed that OCN was expressed by EPCs isolated from the peripheral blood of patients with severe coronary atherosclerosis and calcific aortic stenosis. Furthermore, the number of EPCs expressing OCN was positively correlated with the severity of atherosclerosis [26]. Indeed, CD133+/CD34+/kinase insert domain receptor + cells in peripheral blood are often taken as a sign of unstable atherosclerosis [27]. In postmenopausal women, bisphosphonate treatment downregulates OCN and other osteogenesis-related markers [28]. It is therefore speculated that damage to the vascular endothelium in diabetic patients results in the recruitment of EPCs to the site of damage for the repair of damaged blood vessels. However, if EPCs differentiate into osteoblasts following exposure to harmful substances, such as AGEs, then the resulting atherosclerosis may aggravate vascular injury, leading to severe atherosclerosis.

Most AGEs bind to RAGE to exert their effects within the cell. Studies have shown that the association between AGEs and RAGE can regulate vascular smooth muscle proliferation, promote the infiltration of inflammatory cells, aggravate oxidative stress, induce the calcification of vascular smooth muscle, and exacerbate atherosclerosis [29]. Numerous studies have shown that activation of RAGE by AGEs can promote apoptosis, cellular migration, adhesion, and proliferation in EPCs, as well as weaken the biological activity of EPCs in the repair of blood vessels [30, 31]. The role of AGEs in the development of atherosclerosis remains to be elucidated. However, previous studies have shown that decreased AGE synthesis and reduced binding to RAGE can slow the progression of atherosclerosis in diabetic patients [32, 33]. The results of the present study revealed that the knockdown of RAGE using siRNAs, as well as the inhibition of p38MAPK and JNK signaling, significantly decreased AGE-induced EPC calcification (that is, osteoblast differentiation). These results indicated that EPC calcification may be mediated by p38 and JNK, but not ERK1/2. Decreased binding to RAGE and the inhibition of downstream signaling may prevent endothelial calcification, thus preventing atherosclerosis.

However, our study mainly focused on rat bone marrow-derived EPCs, and further studies are needed to determine whether there are the same changes or mechanisms in different species. Studies by Jia et al. have shown that chemerin (an adipokine that plays an important role in the development of inflammation) can enhance the adhesion and migration abilities of human EPCs and reduce the apoptosis ratio. But in ApoE-/- mice, chemerin can increase lipid accumulation in atherosclerotic plaques and exacerbate plaque instability. This effect is attenuated by specific blocking of...
the P38 MAPK pathway [34]. Therefore, whether human EPCs can differentiate into osteoblasts under the stimulation of AGEs/RAGE and the signal pathway is the focus of subsequent research.

In recent 20 years, studies on the ability of EPCs to protect against atherosclerosis in patients with diabetes have focused primarily on the effects of AGEs on cellular function [35]. As more studies have shown that EPCs can express osteogenic markers, the negative effects of AGEs on the differentiation of EPCs should be considered as well [24]. Additional studies will be necessary to determine how to prevent the differentiation of EPCs into osteoblast-like cells and thus improve the biological ability of EPCs to repair injured blood vessels.

5. Conclusions

Our data indicate that EPCs can differentiate into osteoblasts under certain conditions. The binding of AGEs to their membrane receptor RAGE can promote this process. The underlying mechanism appears to involve the p38MAPK and JNK1/2 pathways, but not the ERK1/2 pathway. This provides a new theoretical basis for the prevention and treatment of vascular complications of diabetes.

Data Availability

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors’ Contributions

Yong Liu and Yanzheng He contributed equally to this work.

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