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Advanced glycation end products impair function of late endothelial progenitor cells through effects on protein kinase Akt and cyclooxygenase-2

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ABSTRACT

Endothelial progenitor cells (EPCs) exhibit impaired function in the context of diabetes, and advanced glycation end products (AGEs), which accumulate in diabetes, may contribute to this. In the present study, we investigated the mechanism by which AGEs impair late EPC function. EPCs from human umbilical cord blood were isolated, and incubated with AGE-modified albumin (AGE-albumin) at different concentrations found physiologically in plasma. Apoptosis, migration, and tube formation assays were used to evaluate EPC function including capacity for vasculogenesis, and expression of the receptor for AGEs (RAGE), Akt, endothelial nitric oxide synthase (eNOS), and cyclooxygenase-2 (COX-2) were determined. Anti-RAGE antibody was used to block RAGE function. AGE-albumin concentration-dependently enhanced apoptosis and depressed migration and tube formation, but did not affect proliferation, of late EPCs. High AGE-albumin increased RAGE mRNA and protein expression, and decreased Akt and COX-2 protein expression, whilst having no effect on eNOS mRNA or protein in these cells. These effects were inhibited by co-incubation with anti-RAGE antibody. These results suggest that RAGE mediates the AGE-induced impairment of late EPC function, through down-regulation of Akt and COX-2 in these cells.

Angiogenesis is an important part of vascular adaptation to ischemia in heart and other organs. Local endothelial cells (ECs) and circulating endothelial progenitor cells (EPCs) play an essential role in this process [1]. EPCs can be isolated, cultured, and differentiated ex vivo from peripheral, bone marrow, and umbilical cord blood mononuclear cells (MNCs) [2]. Two types of EPCs, early and late EPCs, can be derived and identified, and these may play different roles in neovasculogenesis [3,4].

It has previously been reported that EPC function is impaired in diabetes [5,6]. This may be attributable, at least in part, to accumulation of advanced glycation end products (AGEs). These are modified proteins or lipids that become nonenzymatically glycated and oxidized in the presence of aldose sugars. AGEs impair the function of mature ECs in respect of angiogenesis, and accelerate the progression of atherosclerosis, particularly in diabetes [7]. One of its receptors, the so-called receptor for AGEs (RAGE), plays an important role in cell signaling [8]. Whether AGEs affect EPC function is, however, currently unclear.

Angiogenesis is a multistep process, involving extracellular matrix (ECM) degradation, endothelial cell proliferation, migration, and tube formation [9]. Vascular endothelial growth factor (VEGF) plays a crucial role in this process. Following binding of VEGF to its receptor, the phosphatidylinositol 3-kinase (PI3-K)/Akt cascade is activated intracellularly, one of the downstream consequences of which is phosphorylation of endothelial nitric oxide (NO) synthase (eNOS) on Ser-1177, which in turn gives rise to increased endothelial NO biosynthesis [10–12]. NO can enhance matrix metalloproteinase (MMP) activity, especially MMP-2 and MMP-9, thereby augmenting ECM breakdown which is essential for angiogenesis [13,14].

Cyclooxygenase type 2 (COX-2) is another important regulator of cardiovascular homeostasis [15]. In humans, COX-2 is primarily responsible for biosynthesis of the anti-atherogenic, anti-thrombotic, vasodilator prostacyclin (PGI_2) in the vascular endothelium [16]. Vascular expression of COX-2 protein has been found to be substantially increased in the presence of cardiovascular risk factors, including hypercholesterolemia, and in the presence of elevated levels of pro-inflammatory cytokines, oxidized low-density lipoproteins, and hypoxia [17,18]. Whether COX-2 plays any significant role in the cardiovascular pathophysiology of diabetes remains unclear.

The aim of the present study was to examine in detail the effects of AGEs on late EPC function, and to determine the cellular basis of these effects, specifically the role of RAGE, Akt, eNOS, and COX-2.

Materials and methods

Synthesis of AGE-modified albumin. AGE-modified albumin (AGE-albumin) was synthesized under sterile conditions by incubating

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20 mg/ml bovine serum albumin (BSA, low endotoxin, Merck) with 50 mmol/L glucose for 90 days, following which the mixture was fully dialyzed against PBS to remove unbound glucose, as previously described [7]. BSA incubated without glucose under the same conditions was used as the negative control in all experiments.

EPC isolation and culture. The study followed procedures in accordance with ethical standards as formulated in the Helsinki Declaration of 1975 (revised 1983), and was approved by the Institutional Ethics Committee of Nanjing University Medical School. Informed consent was obtained from all umbilical cord donors. Total MNCs were isolated from 50 ml human umbilical cord blood samples from healthy newborns, by density gradient centrifugation with Histopaque-1077 (density 1.077 g/ml; Sigma). MNCs (10^7) were plated in 2 ml endothelial growth medium (EGM-2 MV; Cambrex) on fibronectin-coated (Sigma) six-well plates. After 48 h of culturing, cells unattached were collected and re-plated as before. Thereafter, medium was replaced every 3 days, and each colony/cluster was followed up. Cells at passage 3 (“late EPCs”) were incubated with 50–400 μg/ml AGE-albumin or 400 μg/ml unmodified albumin for 24 h.

EPC characterization. The adherent cells were incubated with 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine–labeled acLDL (DiI-acLDL) (Molecule Probes), and then fixed in 2% paraformaldehyde and counterstained with fluorescein isothiocyanate (FITC)–labeled lectin from ulex europaeus agglutinin (UEA-1) (Sigma). The fluorescent images were recorded under a laser scanning confocal microscope. Cells were also characterized by immunohistochemistry staining for von Willebrand factor (vWF) and immunofluorescence staining for expression of CD45, CD146, and CD105 (Becton Dickinson), followed by detection by fluorescence-activated cell sorter (FACS) analysis (Becton Dickinson).

EPC proliferation assay. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine late EPC proliferation. Passage 3 cells were incubated with 50–400 μg/ml AGE-albumin or 400 μg/ml unmodified albumin for 24 h, following which cells were supplemented with MTT (0.5 mg/ml, Sigma) and incubated for a further 4 h. The blue formazan thus produced was solubilized with dimethyl sulfoxide and absorbance measured at 550–650 nm.

EPC apoptosis assay. After incubation with different concentration of AGE-albumin or unmodified albumin for 24 h, harvested late EPCs were stained with APC-conjugated annexin V and propidium iodide (PI) in binding buffer, for 15 min in the dark. Cells were analyzed by flow cytometry (FACSCanto, Becton Dickinson) using FACSDiva Software (counting 10,000 events per sample). Annexin V^+/PI^- events were taken as representing apoptotic (as opposed to necrotic) cells.

EPCs migration assay. The migratory function of late EPCs was evaluated using a modified Boyden chamber (Transwell, Coster) assay [19]. In brief, 4×10^4 late EPCs were placed in the upper chamber of 24-well Transwell plates with a polycarbonate membrane (8 μm pores) containing serum-free endothelial growth medium; VEGF (50 ng/ml) in medium was placed in the lower chamber. After incubation for 24 h, the membrane was washed briefly with PBS and fixed with 4% paraformaldeyde. The upper side of membrane was wiped gently with cotton wool. The membrane was then stained using hematoxylin solution and removed. Migration of late EPCs was evaluated by measuring the area containing migrated cells as a percentage of the total area, in six random high-power (100×) microscope fields, and the average of these six fields taken.

EPC tube formation assay. ECMatrix gel solution was thawed at 4 °C overnight, then mixed with ECMatrix diluent buffer, and placed in a 96-well plate, at 37 °C for 1 h. Late EPCs (10^4) were placed on matrix solution with EGM-2 MV medium with AGE-albumin or unmodified albumin, and incubated at 37 °C for 16 h. Tube formation was inspected under an inverted light microscope (100×). Four representative fields were taken, and the average of the total area of complete tubes formed by cells was compared by Image-Pro Plus.

Table 1
Sequences of primers and PCR conditions.

	Primer sequence	Product size (bp)	Ta (°C) /cycles
RAGE	S: 5′-CTGGTGTTCCCAATAAGG-3′ AS: 5′-AGGTCAGGGTTACGGTT-3′	372	58.5/38
eNOS	S: 5′-GTTTGTCTGCGGCGATGT-3′ AS: 5′-GTGCGTATGCGGCTTGTC-3′	192	58.0/38
GAPDH	S: 5′-ACCACAGTCCATGCCATCAC-3′ AS: 5′-TCCACCACCCTGTTGCTGTA-3′	452	56.0/26

S, sense; AS, antisense; Ta, annealing temperature.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from each sample using Trizol reagent (Invitrogen) according to the manufacturer’s instructions, and dissolved in DEPC-treated water. The quality of the isolated RNA was checked by agarose gel electrophoresis, and RNA concentration was determined by measuring optical density at 260 and 280 nm. Total RNA (2 μg) in a final volume of 40 μl was subjected to reverse transcription. cDNA synthesis was carried out using random hexamer primers and MMLVreverse transcriptase, under the conditions recommended by the manufacturer (Invitrogen). Specific primers were manually designed using Gene Runner software (Hastings Software, Inc.). Sequences of primers and PCR conditions used are shown in Table 1.

Western blotting analysis. Protein extracts were prepared and Western blotting performed as previously described [7]. The primary antibodies used were monoclonal antibodies to eNOS (1:2000, Becton Dickinson), RAGE (1:1000, Chemicon), COX-2 (1:1000, Chemicon), and Akt (1:1000, Cell Signaling). Bands were visualized by enhanced chemiluminescence detection (Pierce), and densitometic analysis was performed using QuantityOne software (Bio-Rad).

Statistical analysis. All results are expressed as means ± SD of n experiments. Comparisons of 3 or more groups were performed by ANOVA with Dunnett’s post-test. In all cases, $P < 0.05$ (two-tailed) was taken to denote statistical significance. All analyses were performed using SPSS 13.0 software (SPSS, Inc. Chicago, USA).

Results

Characterization of EPCs

Two types of cells were observed in our culture system. One type (“early EPCs”), appeared 3–5 days after primary culture. These early EPCs were spindle shaped and appeared as clusters, but these clusters disappeared in 10–14 days. Another cell population (“late EPCs”) appeared as clusters 10–15 days after plating. Cells (20–50) with smooth cytoplasmic outlines were observed in each cluster, and each cluster gave rise to more than 500 cells, which grew to confluence and exhibited a cobblestone appearance in 1–3 days. More than 95% cells in these clusters were vWF^+ and $CD45^-/CD146^+/CD105^+$, and most exhibited the ability to both endocytose DiI-acLDL and bind FITC-UEA-1 (see Supplement).

AGE-albumin increases late EPC apoptosis and decreases VEGF-induced migration and tube formation

After 24 h synchronization, passage 3 late EPCs were incubated with 50-400 μg/ml AGE-albumin or 400 μg/ml unmodified albumin for 24 h (Fig. 1A). Compared with the unmodified albumin control, AGE-albumin induced a greater degree of apoptosis, as