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Original Article

Caspase-3 expression and cell morphology of early endothelial progenitor cells exposed to N-epsilon-carboxymethyl lysine

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Abstract

The role of endothelial progenitor cells (EPCs) on vascular repair and neovasculogenesis is impaired in diabetes. This study aimed to investigate the effect of N-epsilon-carboxymethyl lysine (CML) on caspase-3 expression and morphology of early EPCs. After 7 days of culturing, primary cultures of early EPCs were randomly divided into four groups. One group is a normal group. Three groups were exposed to low (50 µg/ml), medium (100 µg/ml), and high (200 µg/ml) doses of CML for an hour. The caspase-3 expression and morphology of early EPCs were evaluated by laser scanning confocal microscope. The caspase-3 expression of early EPCs increased in groups exposed to low and medium dose of CML, and decreased in group exposed to high dose of CML compared to normal. Exposure of CML changed the morphology of early EPCs including cells shrinkage, unclear margin between cytoplasm and nucleus, also oval-shape cells. Given that CML may contribute to the functional defects of the endothelium in diabetes by causing early EPCs apoptosis via increased caspase-3 expression.

INTRODUCTION

Endothelial progenitor cells (EPCs) are subset of hematopoietic cells found in bone marrow and circulation [1]. EPCs have stem cell properties, but have limited capacity of differentiation and proliferation. Currently, two types of EPCs, namely early and late outgrowth EPCs, can be derived and identified from peripheral blood. They are unipotent cells that can be differentiated to mature endothelial cells and are involved in formation of new vessels and remodeling of endothelial cells at the site of injury [2-4]. It was recently shown that hyperglycemia reduces the number and function of EPCs. This condition has important roles in pathogenesis of vascular complications in both types 1 and type 2 of diabetes [5-10].

In the pathogenesis of diabetic vascular complications, hyperglycemia induces increased advanced glycation end products (AGEs) formation. It was demonstrated that AGEs induce EPC apoptosis in diabetes [11-14]. Moreover, AGEs impaired late EPC migration and adhesion in a concentration-dependent manner. [14]. N-epsilon-carboxymethyl lysine (CML), a degradation product of Amadori, is a dominant stable AGE compound accumulating in tissues with age, and its rate of accumulation is accelerated in diabetes [15]. The level of CML was shown increased in age of over 60 years compared with less than 45 years [16]. It was

demonstrated that at dose equivalent to serum concentration of CML in end stage renal disease (ESRD) patients without dialysis therapy, CML did not impair late outgrowth EPCs function and did not induce EPCs apoptosis [17]. There is no study explored the effect of CML to apoptosis of early EPCs. It is proved that early EPCs also have important contributions to vascular repair and neovasculogenesis. They are responsible for transient repair preparing the environment that is conducive for more durable vascular repair, which is the function of late outgrowth EPCs [4]. With this background, the present study aimed to investigate the effect of CML on caspase-3 expression and cell morphology of early EPCs.

MATERIALS AND METHODS

Endothelial progenitor cells isolation

Ten ml of EDTA-blood from healthy young volunteers was used to isolate the circulating EPCs. Mononuclear cells (MNCs) were isolated using Ficoll-Paque 1.077 g/ml (MP Biomedicals, Santa Ana, CA, USA) density gradient centrifugation (1600 rpm, 20°C, 30 min), then washed three times with Hanks' solution (Sigma) [6, 10]. The study was approved from the local ethics committee and informed consent was obtained from all subjects.

Endothelial progenitor cells cultivation and N-epsilon-carboxymethyl lysine exposure

MNCs were resuspended in endothelial cell growth medium (M199; Invitrogen) containing 20% fetal bovine serum (Invitrogen), 100 IU/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma), 10 ng/ml human epidermal growth factor (hEGF) (Sigma), and 100 µg/ml endothelial cell growth factor (ECGF) (Sigma). They were then grown on gelatin-coated 48-well plates at 37°C in a 5% CO_2 incubator. Under daily observation, after 3 days of culturing, medium were changed and non-adherent cells were removed; attached early EPCs appeared, elongated with a spindle shape. Thereafter, medium were replaced every 3 days for the entire culture period [6, 10]. After 7 days of culturing, EPCs were incubated with CML (PolyPeptide Laboratories) at doses of 50, 100, or 200 µg/ml in fresh medium at 37°C in a 5% CO_2 incubator for an hour. All experiments were done in triplicate.

Endothelial progenitor cells characterization

The early EPCs were characterized as adherent cells double positive for acetylated low density lipoprotein (Ac-LDL) uptake and lectin binding by direct fluorescent staining. Briefly, the adherent cells were washed gently with medium and incubated with 10 µg/ml 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-labeled Ac-LDL (Dil-Ac-LDL; Biomedical Technologies Inc., Stoughton, MA, USA) at 37°C in a 5% CO_2 incubator for 1 h. Cells then were fixed in 2% paraformaldehyde and counterstained with 10 µg/ml fluorescein isothiocyanate–labeled lectin from Ulex europaeus agglutinin (UEA-1; Sigma) for 45 min [3, 10, 18-20]. Morphology of EPCs was observed under laser scanning confocal microscope.

Analysis of endothelial progenitor cells caspase-3 expression

The protocol was according to the manufacturer's instructions. After treatment, the adherent cells were washed gently with phosphate buffered saline (PBS) and fixed in 2% paraformaldehyde. Cells were incubated with 20 µl/ml caspase-3 antibody (Santa Cruz Biotechnology, Dallas, TX, USA) for 1 h, and then incubated with 10 µl/ml phycoerythrin-fluorescent dye (Santa Cruz) for 45 min. The caspase-3 expression was evaluated by counting fluorescent intensity of cells (in arbitrary units; AU) in five random high-power (x400) microscope fields. The fluorescent images were recorded under a laser scanning confocal microscope.

Statistical Analysis

Data are presented as means ± SD. Intergroup comparisons were performed by one-way analysis of variance (ANOVA). Probability values of $P < 0.05$ were considered statistically significant.

RESULTS

Endothelial progenitor cells characterization

As previously described [3], the peripheral blood MNCs that initially seeded on gelatin-coated wells were round (Fig.1A). After changing medium on day 4, attached early EPCs formed characteristic colony. EPCs colony-forming unit was identified as a central core of round cells with elongated sprouting cells at the periphery (Fig.1B). On day 14, late EPCs with cobblestone-like morphology similar to mature endothelial cells were grown to confluence (Fig.1C). EPC colony on day 7 was further confirmed as cells double positive for Dil-Ac-LDL uptake (Fig.1E; red color) and UEA-1 binding affinity (Fig.1F; green color).

We also observed characteristic morphological changes of early EPCs exposed to CML including unclear of margin between cytoplasm and nucleus, cytoplasmic blebs, and apoptotic bodies (Fig.2D-F). Thus, cells were indicated undergoing apoptosis.

Endothelial progenitor cells caspase-3 expression

The exposure of several doses of CML to early EPCs affected the caspase-3 expression, as shown in Fig.3. There was increased caspase-3 expression in early

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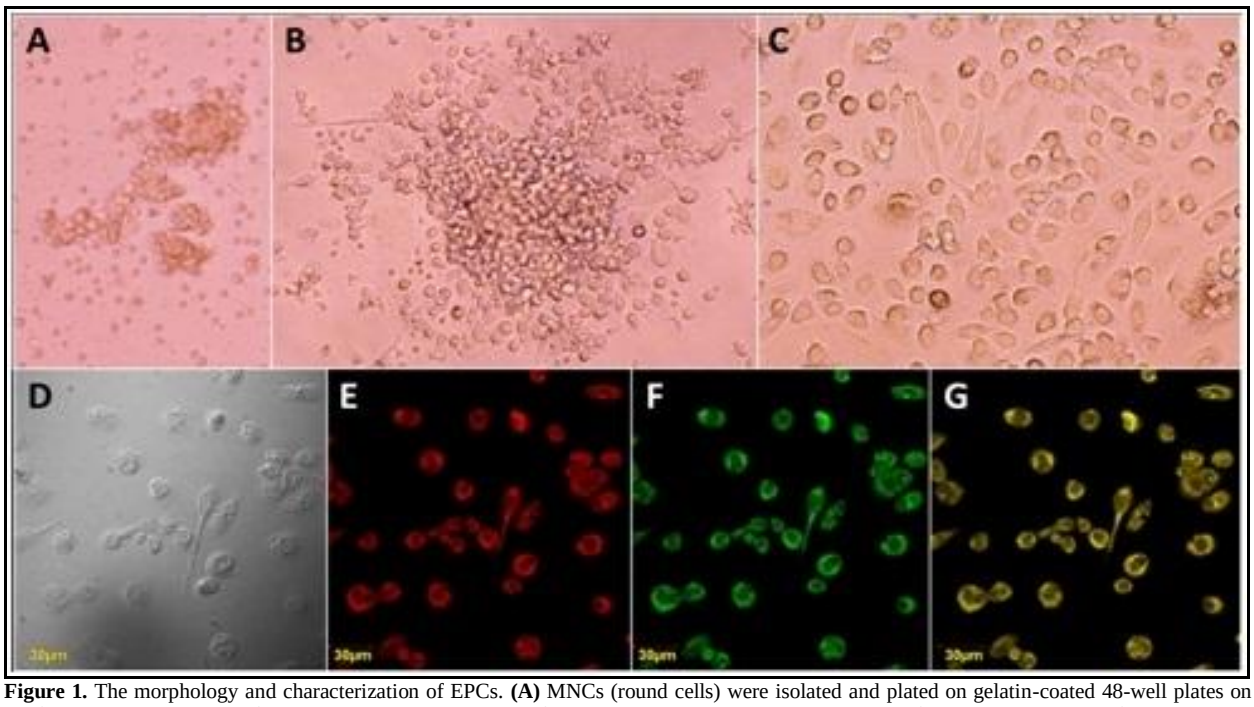

Figure 1. The morphology and characterization of EPCs. **(A)** MNCs (round cells) were isolated and plated on gelatin-coated 48-well plates on the first day; **(B)** Four days after plating, adherent early EPCs formed characteristic colony. EPCs colony-forming unit was identified as a central core of round cells with elongated sprouting cells at the periphery; **(C)** Fourteen days after plating, late EPCs with cobblestone-like morphology were grown to confluence; **(D)** Differential interference contrast **(**DIC) of early EPCs; **(E)** Cells were shown to uptake Dil-Ac-LDL (red fluorescent); **(F)** Cells were shown to bind UEA-1 (green fluorescent); **(G)** Double positive cells (yellow fluorescent).

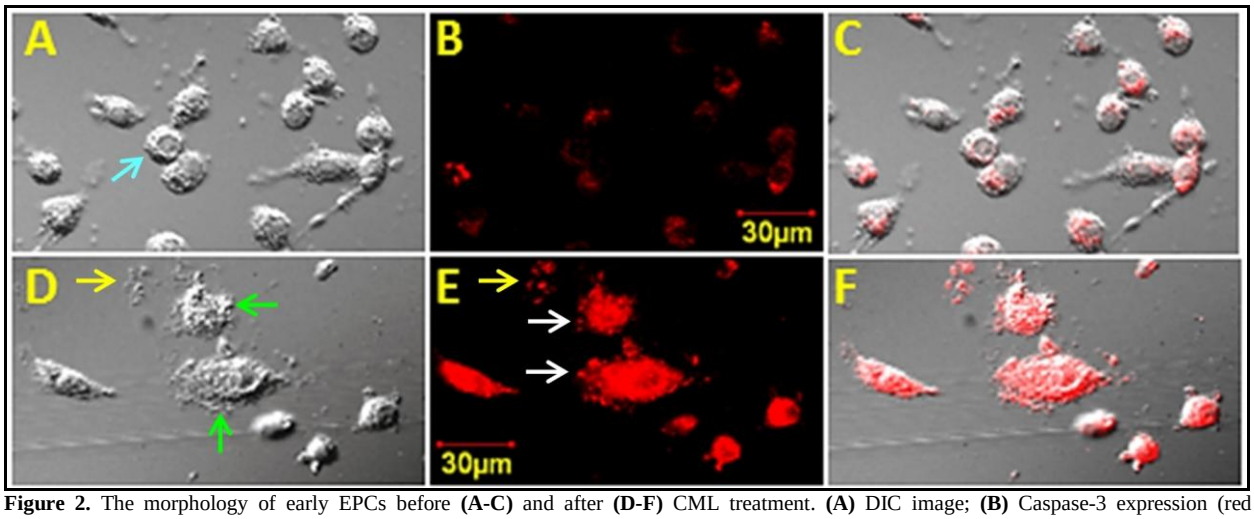

Figure 2. The morphology of early EPCs before **(A-C)** and after **(D-F)** CML treatment. **(A)** DIC image; **(B)** Caspase-3 expression (red fluorescent); **(C)** Superimposed image of normal early EPCs. The margin between cytoplasm and nucleus of normal cells are clear (blue arrow). **(D)** DIC image; **(E)** Caspase-3 expression (red fluorescent); **(F)** Superimposed image of early EPCs exposed to 100 μg/ml of CML. Cells have unclear of margin between cytoplasm and nucleus (green arrows), cytoplasmic blebs (white arrows), and fragmentations into a number of small apoptotic bodies (yellow arrows); these are characteristic of apoptotic cells.

EPCs exposed to low (50 μg/ml; 891.25 ± 379.52 AU) and medium (100 μg/ml; 1321.15 ± 200 AU) dose of CML compared to normal (650.13 ± 18.43 AU, $P < 0.05$). Interestingly, high dose (200 μg/ml) of CML exposure induced a similar level (978.19 ± 392.13 AU) of early EPCs caspase-3 expression as low dose exposure, but less than medium dose ($P < 0.05$).

DISCUSSION

The present study showed that primary culture of early EPCs from peripheral blood revealed several biologic markers, including double positive of Dil-Ac-LDL and UEA-1. This finding is consistent with previous studies [3-4, 21]. Early EPCs, which are the cells originally identified by Asahara *et al* [22], are mainly derived

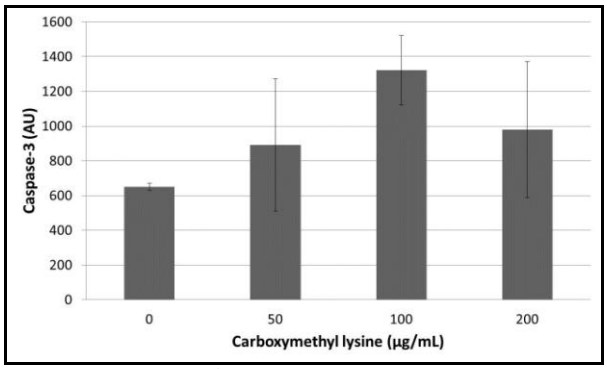

Figure 3. The levels of caspase-3 expression in EPCs exposed to CML. The highest EPCs caspase-3 expression were achieved at the dose of 100 µg/ml, and then decreased at the dose of 200 µg/ml.

from monocytes, do not proliferate and begin to gradually die after a few weeks in culture [4, 23]. In contrast, late outgrowth EPCs usually begin growing later than 2 weeks after isolation, proliferate very rapidly, and resemble endothelial cells (with cobblestone morphology) [4, 24]. Early EPCs are the key cells responsible for "provisional repair", that is a transient repair preparing the environment for more "durable repair", which is the function of late outgrowth EPCs. It is shown that in healthy retina both populations are participating in an ordered, temporal sequence with early EPCs homing in first, attracting the late outgrowth EPCs later. It is also demonstrated in non-proliferative diabetic retinopathy that reduced function of early EPCs impairs recruitment of late outgrowth EPCs leading to ineffective repair of damaged capillaries and the development of acellular capillaries [4]. It is proved that early EPCs also have important contributions to vascular repair and neovasculogenesis.

Apoptosis is a programmed cell death triggered by many signals [25], including physiologic (intrinsic) stimulus and extrinsic stimulus. There are three steps in apoptosis; ***(1)*** initiation or stimulus phase for cell death; ***(2)*** effector or commitment phase which is time to deciding cell suicide; and ***(3)*** degradation or execution phase when cell shows the biochemistry and morphology of apoptosis [26]. Caspases are the key enzymes that cause cell apoptosis. Once the signal pathway of caspase-3 is activated, proteins inside cells can be degraded and cell apoptosis irreversibly occurs. Caspase-3, as an important member of the caspase family, is the executor of cell apoptosis. The execution pathway results in characteristic cytomorphological features including cell shrinkage, chromatin condensation, formation of cytoplasmic blebs and apoptotic bodies and finally phagocytosis of the apoptotic bodies by adjacent parenchymal cells, neoplastic cells or macrophages [27]. Several previous studies showed that AGEs inhibit proliferation of $CD34^+$ cells at dose 2 or 20 µg/ml and induce apoptosis of EPCs at dose 200 µg/ml [11] or 200 mg/ml [13]. The present study demonstrated biphasic response of caspase-3 expression in early EPCs exposed to several dose of CML, the dominant stable AGE compound in tissue proteins. Low and medium dose of CML increased caspase-3 expression, but high dose of CML decreased caspase-3 expression in early EPCs. We also found morphological changes of early EPCs exposed to CML including cell shrinkage, unclear of margin between cytoplasm and nucleus, and oval-shape cells. These indicated that cells undergo apoptosis.

The increased caspase-3 expression in early EPCs exposed to CML is consistent with previous studies in fibroblast apoptosis [28, 29]. CML is a known ligand for AGE receptors (RAGE), thus increased caspase-3 expression in early EPCs may be due to CML-induced activation of RAGE [30]. Interaction of CML-RAGE has been reported to stimulate several signaling pathways. Previous study demonstrated that CML-rich collagen increases the formation of intracellular reactive oxygen species (ROS), nitric oxide (NO), and ceramides leading to p38 and c-Jun NH(2)-terminal kinase (JNK) mitogen-activated protein (MAP) kinase activation, which in turn induces forkhead box protein (FOX)O1 and caspase-3. FOXO1 activation is induced by p38 and JNK, which in turn, depend on the formation of ROS. Thus p38 and JNK are important mediators of FOXO1 induced by CML-rich collagen, and FOXO1 activation represents an important mechanism through which the MAP kinase signaling pathway induces cell death. These events are critical, since inhibition of each significantly reduces apoptosis induced by CML-rich collagen [29]. Previous study also showed that CML-rich collagen activates caspase-8, which signals apoptosis through the cytosolic pathway, and, to a lesser extent, caspase-9, which signals through the mitochondrial pathway. Consistent with the degree of activation, inhibition of caspase-8 *in vitro* had a greater impact than inhibition of caspase-9. Study also showed that both of these pathways stimulated activation of caspase-3. The involvement of caspase-3 in AGE-stimulated apoptosis was shown by enhanced caspase-3 activity and through inhibition of apoptosis with a specific caspase-3 inhibitor [28]. Furthermore, CML-rich collagen stimulated a global induction of pro-apoptotic genes that involved several classes of molecules: ligands, receptors, adaptor molecules, mitochondrial proteins, and caspases [28, 29]. High level of oxidative stress induced by CML may also damage and disrupt the mitochondrial electron transfer leading to induction of apoptosis [31, 32]. We also found characteristic morphological changes of early EPCs exposed to CML including unclear of margin between cytoplasm and nucleus, cytoplasmic blebs, and apoptotic bodies. These

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indicated that cells undergo apoptosis. Given that diabetes via CML may contribute to the functional defects of the endothelium in diabetes by causing early EPCs apoptosis via increased caspase-3 expression. Furthermore, an understanding of the signaling mechanisms may provide therapeutic targets that are ultimately of clinical benefit.

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COMPETING INTERESTS

None declared

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