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Homocysteine inhibits endothelial progenitor cells proliferation via DNMT1-mediated hypomethylation of Cyclin A

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ABSTRACT

Endothelial progenitor cells (EPCs) contribute to neovasculogenesis and reendothelialization of damaged blood vessels to maintain the endothelium. Dysfunction of EPCs is implicated in the pathogenesis of vascular injury induced by homocysteine (Hcy). We aimed to investigate the role of Cyclin A in Hcy-induced EPCs dysfunction and explore its molecular mechanism. In this study, by treatment of EPCs with Hcy, we found that the expression of Cyclin A mRNA and protein were significantly downregulated in a dose-dependent manner. Knockdown of Cyclin A prominently reduced proliferation of EPCs, while over-expression of Cyclin A significantly promoted the cell proliferation, suggesting that Hcy inhibits EPCs proliferation through downregulation of Cyclin A expression. In addition, epigenetic study also demonstrated that Hcy induces DNA hypomethylation of the Cyclin A promoter in EPCs through downregulated expression of DNMT1. Moreover, we found that Hcy treatment of EPCs leads to increased SAM, SAH and MeCP2, while the ratio of SAM/SAH and MBD expression decrease. In summary, our results indicate that Hcy inhibits Cyclin A expression through hypomethylation of Cyclin A and thereby suppress EPCs proliferation. These findings demonstrate a novel mechanism of DNA methylation mediated by DNMT1 in prevention of Hcy associated cardiovascular disease.

1. Introduction

Homocysteine (Hcy) is an independent risk factor of atherosclerosis (AS) [1–3]. Endothelial dysfunction is a key feature of AS, which induces platelet aggregation, activation of coagulation, vascular smooth muscle cells (VSMCs) proliferation, and it initiates the pathogenesis of AS [4–6]. Endothelial progenitor cells (EPCs) are considered as precursors of mature endothelial cells (ECs), they originate from bone marrow and migrate to the sites of vascular damage to re-establish an intact endothelial layer following the denudation of the endothelium [7]. It is believed that the damaged endothelium can not only be repaired by the proliferation and migration of neighboring ECs, but also by EPCs [8]. Therefore, it is necessary to elucidate the pathological mechanism of EPCs dysfunction induced by Hcy for better understanding of AS.

Cyclin A (also named Cyclin A2 as opposed to the male germ cell–specific CyclinA1) is a pivotal regulator of cell cycle progression in the onset of both DNA replication and mitosis [9]. Inhibition of Cyclin A by microinjection of antibodies against Cyclin A or by antisense Cyclin A RNA can suppress DNA synthesis and mitosis [10]. Deficiency of Cyclin A leads to early death of murine embryo [11]. In addition, precautious expression of Cyclin A in G1 cells accelerated their S-phase entry, indicating that Cyclin A is controlled mainly at the transcriptional level [12]. In ECs, clinically relevant concentrations of Hcy (10-50μmol/L) induce cell cycle arrest at G1/S transition via inhibition of Cyclin A transcription [6]. It is interesting to know whether Hcy regulates EPCs proliferation through Cyclin A as well.

Association between Hcy and aberrant DNA methylation has been reported in several diseases including AS, osteoporosis, uremia and alcoholism [13]. DNA methylation occurs mainly at CpG islands in a

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Table 1
Primer sequences for real-time quantitative PCR (qRT-PCR) analysis.

Gene	Genbank	primer sequence (5′→3′)	Length (bp)	Annealing temperature
Cyclin A	NM003102	Forward primer: TATTCGGGACTCTGAGGGCG Reverse primer: GTCTCACCTTCGCCTTTGCT	244	62.3 °C
DNMT1	NM001379	Forward primer: CTACCAGGGAGAAGGACAGG Reverse primer: GCTACACCGCAGACACTC	152	53.3 °C
MBD	NM004992	Forward primer: AAGTGGAGTTGATTGCGTAC Reverse primer: TTGGTGGATTCTTCGGGT	126	53.2 °C
MeCP2	AF331856	Forward primer: CTGGGTCATGTGGTTCGG Reverse primer: TCTAATAACTACTCGCGTGT	448	58.5 °C

gene, and the extent of DNA methylation is determined by the balance between methylation and demethylation [14]. In mammalian cells, DNA methylation is catalyzed by three DNA methyltransferases (DNMTs): DNMT1, DNMT3a, and DNMT3b. DNMT1 is the most abundant DNMT in mammalian cells and it is responsible for the routine methylation maintenance throughout the life of an organism, whereas DNMT3a and DNMT3b are de novo DNMTs that act to set up DNA methylation patterns early in development [15]. Hcy is a product of the methionine cycle, it is involved in one-carbon methyl group-transfer metabolism and acts as a methyl donor when it is converted to S-adenosyl-methionine (SAM) [16]. SAM can be converted into S-adenosyl homocysteine (SAH), and which is a potent inhibitor of SAM-dependent methyltransferases, thereby it causes cellular hypomethylation. The intracellular SAM/SAH ratio has been considered as an indicator of cellular methylation capacity [17].

In the present study, we aimed to investigate whether Hcy can regulate the expression of Cyclin A and underlying mechanism in Hcy-induced inhibition of EPCs proliferation. We found that Hcy induces hypomethylation of Cyclin A promotor, and thereby inhibits Cyclin A expression due to decreased expression of DNMT1. Our results provide novel insights into the molecular mechanism underlying Hcy-induced endothelial dysfunction.

2. Materials and methods

2.1. Isolation and identification of EPCs

Blood samples from abdominal aorta of rats were collected in tubes containing ethylene diamine tetraacetic acid (EDTA) and further processed within 24 h. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation with ficoll separating solution (Sigma-Aldrich, St. Louis, MO, USA). Isolated cells were washed twice with phosphate-buffered saline, re-suspended in phosphate-buffered saline and inoculated on the plates with pre-coated Human Fibronectin (HFN) (R&D Systems, Minneapolis, MN), and cultured in DMEM high glucose medium for 5 days. The culture medium which contains vascular endothelial growth factor, insulin-like growth factors and fibroblast growth factor was replaced every 24 h. To quantify circulating EPCs in the isolated mononuclear cells, cells were labeled with CD34-FITC antibody (ebiosciene, United States), CD133-PE antibody (ebiosciene, United States) followed by analysis on FACS Vantage SE (Becton Dickinson and R&D systems).

2.2. Cell proliferation assay

MTT assay (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was used to determine EPCs proliferation. Cells were cultured in 96-well plate at a density of 1×10^4 cells/well. After treatment with different concentrations of Hcy (0, 50, 100, 200, 500 μmol/L Hcy, 500 μmol/L Hcy + 30μmol/L folate + 30μmol/L vitamin B12, or 500 μmol/L Hcy + 30μmol/L) 5-azacytidine (AZC), cells were incubated with MTT (0.5 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) for 4 h. After addition of Dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA), the absorbance was measured at 490 nm.

2.3. Migration of EPCs

Boyden chambers (QiLin Beier Laboratory equipment manufactory Co., Ltd, China) with 8-μm pore size polycarbonate filter inserts for 24-well plates were used according to the manufacturer's instructions. Briefly, cells (1×10^6/mL) in 200 μL of DMEM medium with 8% FBS were seeded in the upper chambers with different concentrations of Hcy, the chambers were placed into wells containing 800 μL of complete medium to induce cell migration. The migration chambers were incubated for 24 h at 37 °C. After incubation, the number of cells that migrated into the wells was counted. Each assay was performed in duplicate and repeated three times.

2.4. Detection of SAM and SAH concentrations by high-performance liquid chromatography (HPLC)

SAM and SAH concentrations were measured by HPLC. The supernatant of each sample was filtered through 0.22 μm filter (millipore) and then loaded into a C18 column (250 mm × 4.6 mm ID, 5 μm particle), run by a water HPLC system and connected to an ultraviolet detector. Absorption of eluted compounds was monitored at (lambda) ex = 254 nm. Chromatograms were recorded by a D-2000 Elite HPLC integrator with its quantification accomplished by automatic peak area integration. SAM and SAH standards were used to identify the elution peaks. All analyses were performed in triplicate.

2.5. Real-time quantitative PCR (qRT-PCR)

The total RNA was isolated by Trizol reagent (Invitrogen; Thermo Fisher Scientifc, Inc.). The primer sequences of Cyclin A, DNMT1, MBD and MeCP2 are listed in Table 1. cDNA was synthesized by the Revert Aid first strand cDNA synthesis kit (Thermo Scientific, Glen Burnie, USA). The real-time PCR was carried out by an FTC2000 real-time PCR detection system (Funglyn, Toronto, Canada) with the program as following: 95 °C for 45 s, 55.3 °C for 45 s and 60 °C for 60 s, run 40 cycles. The RNA level of each gene was acquired from the value of the threshold cycle (Ct) of the real-time PCR as related to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) through Equation 1: Ct = Ct (GAPDH) − Ct (sample). Final results were expressed as N-fold differences in the target gene expression and being relative to the calibrator termed “Ntarget”, they were determined as Equation 2: $\text{Ntarget} = 2^{\text{Ct(sample)} - \text{Ct(calibrator)}}$, where Ct values of the calibrator and sample were determined by subtracting the Ct value of the target gene from the Ct value.

2.6. Western blot

The cells were lysed in a lysis buffer (Invitrogen; Thermo Fisher Scientifc, Inc.) and centrifuged at 16000 × *g* at 4 °C for 30 min.