The effects of Hcy on the expression and the methylation status in promoter region of estrogen receptor α gene

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Abstract: To investigate the impacts of Homocysteine on the expression and DNA methylation in promoter region of estrogen receptor α (ERα) gene, and explore its potential mechanism involved in the pathogenesis of atherosclerosis. Cultured smooth muscle cells (SMCs) of humans and THP-1 monocytes were treated by Hcy with different concentrations for different periods of time. The DNA methylation status was assayed by nested methylation-specific polymerase chain reaction, the expression of ERα of the cells was detected by immunohistochemical assay. In the two kinds of cells, the results all showed that the Hcy treatment resulted in de novo methylation in the promoter region of the ER gene with a concentration- and treating time-dependent manner, and the hypermethylation of the ERα gene lead to the decreased expression of ERα. These data indicated that Hcy had the function of inducing de novo methylation in the promoter region of the ER gene of THP-1 cells and SMCs. The atherogenic mechanism of Hcy might involve the hypermethylation of the ER gene, leading to the proliferation of SMCs in atherosclerotic lesions.
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1. Introduction

DNA methylation is an important epigenetic process leading to the chemical modification of genome that plays a critical role in gene regulation (Friso S et al., 2002). The addition of methyl group to cytosine mainly located in CpG nucleotides pairs represents one of the mechanisms by which the genome can behave as a 'responsive organ' for environmental factors. Accumulating evidences have shown aberrant DNA methylation patters in various sets of diseases, including cancer, certain X-linked genetic diseases, autoimmune diseases, aging **etc** (Huang YS et al., 2007). Up to now, more and more evidences shown that the estrogen receptor α (ERα) has aberrant hypermethylation in its promotion region in atherosclerosis development(Yao J et al., 2009).

ERα is a potential growth suppressor gene (Dong CM et al., 2002), in vivo and in vitro models of vascular disease, have found estrogen to be protective against SMC proliferation and neointima formation (Russell R, 1993) clinical evidences also strongly suggested that estrogen replacement therapy in postmenopausal women might help prevent cardiovascular disease(Schwartz SM et al., 1995). Immunologic derangements contribute to disrupt cholesterol balance of monocytes/macrophages in the artery wall is a common phenomenon of premature atherosclerotic cardiovascular disease (ASCVD). The selective inactivation of ERα isoforms through the promoter CpG methylation pathway was demonstrated in leukemia (Hulley S et al., 1998).

Homocysteine (Hcy) is one of the established risk factors for atherosclerosis. So how is the impact of Hcy on the methylation patter of ERα in the SMCs and THP-1 cells? We hence designed the current study to investigate the potential effects of homocysteine with various concentrations and treating times on methylation patter of ERα gene and the expression of ER in the SMCs and THP-1 cells.

2. Material and Methods

Materials

DMEM/F12 medium and RPMI 1640 were purchased from Gibco/Life Technology (Burlington, Ont, Canada). Homocysteine, PMA, sodium bisulfite and hydroquinone all were obtained from Sigma–Aldrich (St. Louis, MO, USA). Neonatal Bovine Serum were from Sijiqing Biotechnologn Corporation (Hangzhou, China). Mouse Anti-α actin Ab was from Beijing Zhong Shan Golden Bridge Biotechnogn Corporation(Beijing, China). Total DNA Extraction kit, agarose gel DNA fragment recovery kit, Boracker reverse transcription-polymerase chain reaction (RT-PCR) Kit and BIOZOL Reagent all were from Tianggen Biotech

corporation (Beijing, China). DNA mate and Methylase AluI were from TaKaRa Biotechnology Co., Ltd (Dalian, China).

Cell culture and treatment

Freshly human umbilical cords were obtained with informed consent from women with normal pregnancies undergoing abdominal delivery in West China Second Hospital at Chengdu. Approval was granted by the Sichuan province ethics committee. Sampling of umbilical cord vessels was processed on the day of delivery.

Umbilical vein smooth muscle cells were prepared by explant technique. After removal of the endothelium and adventitia, the remaining tissue was cut into small pieces, which were planted onto tissue culture flask using Pasteur-pipette, and then bathed in DMEM/F12 medium supplemented with 20% neonatal bovine serum, 2 mmol/L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cultures were maintained at 37°C in 5% CO_2 humid atmosphere. Monolayer confluent VSMC between 3–5 passages were used for all experiments. Cells were identified as VSMC by positive staining with smooth muscle cell specific [alpha]-actin antibody, a marker of smooth muscle cells.

The THP-1 monocytes (From the department of immunology, Sichuan University) were cultured in RPMI 1640 supplemented with 10% heat-inactivated f et al calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37℃ in 5% CO_2 humid atmosphere.

Confluent (85–95%) HUSMCs and THP-1 cells were washed thrice with PBS before the experiments. Then the cells were treated with 50,100,200,500,1000μM Hcy in serum-free DMEM/F12 medium for 24, 48, 72h respectively.

DNA extraction and sodium bisulfite treatment

DNA extraction and the treatment with sodium bisulfite were carried out as previously described(Hulley S et al., 1998). Briefly, the cultured cells were collected, and washed for 3 times with PBS. Total DNA was extracted from cells using Total DNA Extraction kit, according to the manufacture's protocols.

Ten micrograms of DNA in 50 μl of Tris-EDTA Buffer were denatured with 5.5 μl of 3M NaOH at 37 ºC for 10 min, followed by a 16h treatment at 50 ºC after adding 30μl of freshly prepared 10 mM hydroquinone and 520 μl of freshly prepared 3.6 M sodium bisulfite (pH 5.0). The DNA was desalted using a home dialysis system with 1% agarose, then incubated at 37 ºC for 15 min with 5.5 μl of 3NaOH, followed by ethanol precipitation with 33 μl of 3M NaAC (pH 5.2), 4 μl of DNA mate and 300 μl of ethanol. After washing with 70% ethanol, the gently dried DNA pellet was dissolved with 30 μl of Tris-EDTA Buffer at 65ºC for 10 min. The DNA sample was immediately stored at −20 ºC until further use.

Nested Methylation-Specific-Polymerase Chain Reaction (nMS-PCR)

nMS-PCR was used for the dectection of methy-lation in the promoter regions of ER gene. nMS-PCR consists of two-step PCR amplifications after a standard sodium bisulfite DNA modification in which unmethylated cytosine residues are converted to thymine, whereas methylated cytosine residues are retained as cytosine at CpG sites., then utilises this difference to specifically amplify either methylated or unmethylated DNA.The first step of nMSP uses an outer primer pair set that does not contain any CpG. The second-step PCR was performed with the conventional MSP primers. Primers for the promoter region of ER gene were designed to include eight CPG dinucleotides that have benn linked to regulation of ER gene expression. The summary of the primers, product sizes of the MSP assays are shown in Table 1 (Table 1).

To reduce mispriming and to increase efficiency, touch-down(TD) PCR was employed in the amplification. Following hot start, samples were subjected to 20 cycles in a TD program (94 ℃for 45s, annealing temperature for 45s and 72℃ for 45s for 20 cycles, followed by a 1℃ decrease of the annealing temperature every second cycle).After completion of the TD program, twenty cycles were subsequently run (94 ℃for 45s, 45℃ for 45s and 72℃ for 45s), ending with a 5 min extension at 72℃.

The PCR products were separated by electrophoresis through a 1% agarose gel containing ethidium bromide. DNA bands were visualized by ultraviolet light.

The DNA samples of HUSMCs and THP-1 cells without any treatment were used as the unmethylated control, and the methylated control DNA targets were prepared through treating DNA with Methylase AluI that methylates cytosine residues within all CpG dinucleotides in vitro.

PCR products were gel purified with agarose gel DNA fragment recovery kit according to the manufacturer's instruction and were sequenced by Invitrogen Co., Ltd(Shanghai, China).

Immunohistochemistry

ER expression was demonstrated with the avidin-biotin peroxidase complex method using monoclonal anti-ER antibody. After the cover slip with cells was washed with 0.01 M phosphate-buffered saline (PBS) for three times, the endogenous peroxidase was blocked with 3% H2O2 for 30 min, and then the cover slip were washed with 0.01 M

PBS three times, placed in a thermoresistant plastic box filled with 10 mM, pH6.0 citrate buffer, and heated to boiling in a microwave oven, natural cooling, washed with PBS two times. The cover slip were then incubated with bovine serum albumin for 20 min at room temperature, discard unnecessary fluid, covered anti-ER monoclonal antibody overnight at 4℃ in a humid chamber. After washed with PBS three times for 2 min, the cover slip were added 1 drop of biotinylated IgG secondary antibody for 20 min. The cover slips placed in humid chamber and incubated for 20 min at room temperature, then washed with. After the cover slip were washed in PBS three times for 2 min, 3,3'-diaminobenzidine tetrahydrochloride solution with H_2O_2 (0.03%) was used for visualization.Methyl green was used as a counterstain for nuclei.

Data analysis

The data were analyzed using the software SPSS 12.0 for Windows. Data were presented as mean ± SD. For comparison between multiple groups, quantitative data were analyzed using one-way ANOVA and LSD test, and qualitative data were analyzed using chi-square test. Values which were less than $P < 0.05$ were considered significant.

Table 1. Sequence,location and characterization of the primers used in PCR amplification

Primer set	Primer sequence (5'-3')	Seq No	Size (bp)	Annealing temperature
ER-n	GAGGTGTATTTGGATAGTAG	2460	422	63
	AACTCCCTAAACTCTCCCTT	2881		
ER-M	CGTCGTGTATAATTATTTCGAGGGC	2486	283	65
	CTCGCGCACCGTATAACCGCTAAAC	2770		
ER-U	TGTTGTGTATAATTATTTTGAGGGT	2486	283	65
	CTCACACACCATATAACCACTAAAC	2770		

note：ER-n：nested prime；ER-U：unmethylation primer；ER-M：methylation primer

3. Results

Alterations of methylation status of ER gene in HUSMCs treaed by ox-LDL and Hcy with different concentrations and different treating time

Figure 1 displayed the results of methylation status of ERα gene with different treatment.

Figure A-C illustrated the effect of Hcy with different concentrations and different treating time, the results exhibited significant dose-dependent and treating time-dependent de novo methylation in the promotion region of ERα gene. Along with the prolongation of treating time and increased Hcy concentration, the methylated bands became more and more strong, while the unmethylated bands became weaker and weaker, in 72h treating group the unmethylated bands were totally lost, just left methylated bands.

The above results indicated that Hcy could exert the effects on atherosclerosis development through inducing abberant methylation in promotion region of ERα gene, generally in hypermethylation.

U indicated the unmethylated PCR band with primers matching the sequences that the cytosine in giving DNA sequences were unmethylated, which would be converted to thymidine after sodium bisulfite treatment.

M indicated the methylated PCR band with primers matching the sequences that the cytosine in given DNA sequences were methylated, which would not be influenced by sodium bisulfite treatment, remained to be cytosine.

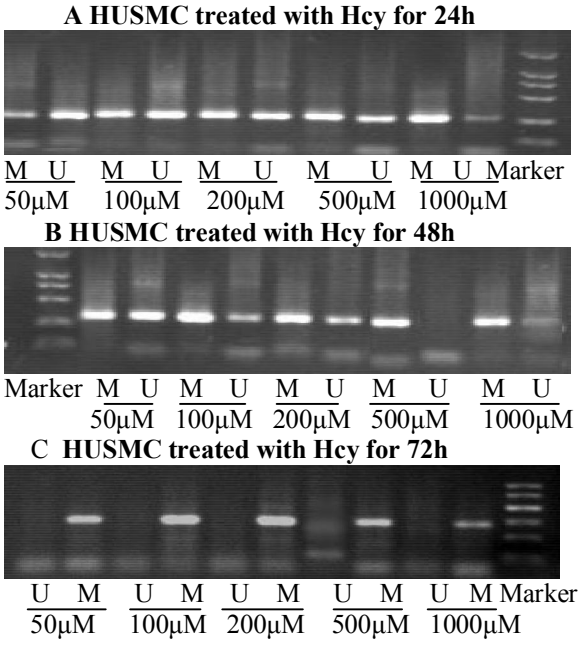
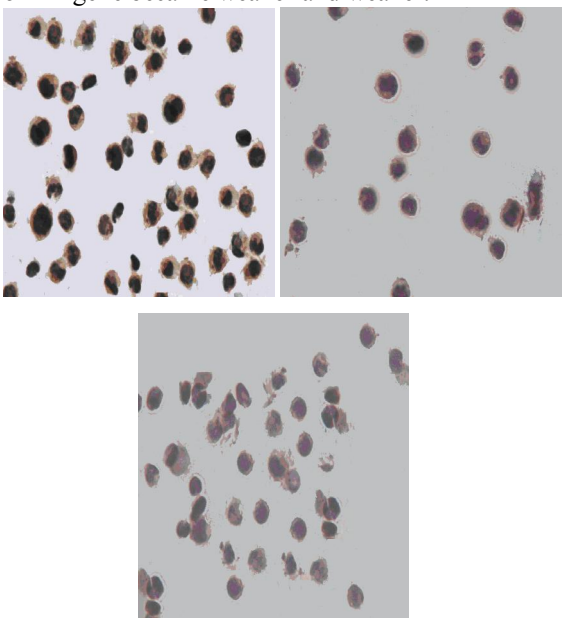


Figure 1. The alteration of methylation status of ERα gene treated by Hcy with different concentration and different treating time.

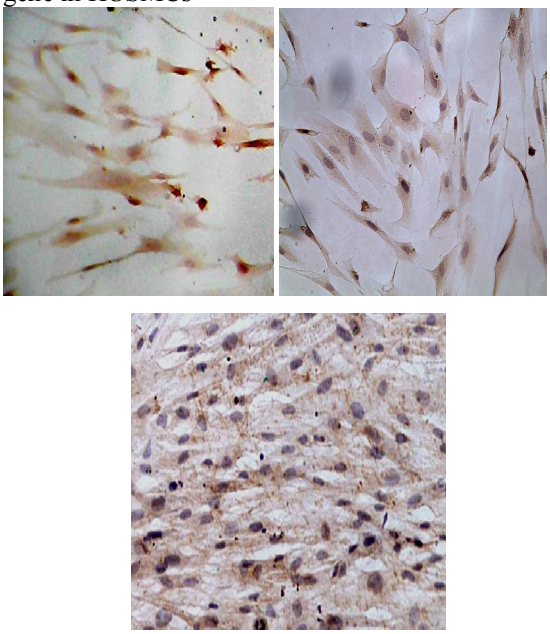
Expression of ER gene in HUSMCs and THP-1 cells treaed by 200μM Hcy with different treating time

The results showed that Hcy could inhibit the expression of ER gene in two kinds of cells. Along with the prolongation of treating time, the expression of ER gene became weaker and weaker.



The control group(200×) 200μM Hcy for 48h(200×) 200μM Hcy for 72h(200×)

Figure 3 the immunohistochemistry results of ER gene in HUSMCs



The control group(200×) 200μM Hcy for 48h(200×) 200μM Hcy for72h(200×)

Figure 2. The immunohistochemistry results of ER gene in THP-1 cells

4. Discussions

Atherosclerosis is a disease of large and medium-sized arteries and is characterized by lipid accumulation and smooth muscle cell (SMC) migration and proliferationp (Ylä-Herttuala S et al. 1986 and Benditt EP et al.1973). Multiple mechanisms have been referred to implicate in its pathogenesis. The earliest recognized gross lesion in atherogenesis is the fatty streak, characterized by an accumulation of cells loaded with cholesteryl esters (foam cells) just beneath the endothelium.

ERα, upon activation by estrogen, regulates a variety of cellular activities, including the inhibition of cell proliferation, such an anti-proliferative effect also involved the proliferation of SMCs(Anita K et al., 2000) To date, ERα represents the only gene known to have differential CpG island methylation in its promotion region in atherosclerosis. In atheromas , ERα methylation was significantly increased when compared with normal proximal aortas (Post WS et al. 1999)

Homocysteine, when concentration elevated in plasma, has been considered an independent risk factor for cardiovascular disease. The atherogenic mechanism of hyper-homocysteinemia may involve varieties of effects, including causing vascular endothelial dysfunction/injury, attenuation of NO-mediated vasodilatation, disturbance in the antithrombotic activities of the endothelium, oxidative stress, activating Nuclear Factor-B and leading to recruitment of leukocytes and monocytes etc (Kathy KW et al. 2004 and Sanjana D et al.2001). Homocysteine is also intimately associated with S-adenosylmethionine (SAM), the methyl donor for more than 100 differnent transmethylation reaction including DNA methylation (Guilland JC et al. 2003 and Hercberg L et al.1999) So hyperhomocysteine may interfere with the epigenetic modification of genome. Hiltunena et al have reported that a genomic hypomethylation occurred during atherogenesis in human, mouse and rabbit lesions and MTase was expressed in atherosclerotic lesions (Mikko O et al. 2002). Altered gene expression and cell proliferation in atherosclerotic lesions have some similar characteristics with certain solid tumors, which show genomic hypomethylation and hyper-methylation in some tumor suppression genes. Our research group has recently also found that Hcy could increase the activity of methyltransferase (data to be published). So maybe Hcy could exert its atherogenic effect through the way of inducing aberrant methylation pattern in ERα gene.

In fact, the data in present study demonstrated a dose-dependent de novo methylation in the promotion region of ERα gene along with the

increasing homocysteine concentration and treating time, and we found that hypermethylation status of promoters could inhibit the ERα expression. This result potentially suggests that the de novo methylation in the promotion region of ERα gene, induced by Hcy may involve in the mechanism of SMC proliferation in atherogenesis.

In summary, our experimental results indicated that Hcy could cause abberant methylation in promotion region of ERα gene, while the Hcy-induced hypermethylation in the promotion region of ERα gene showed similar dose-dependent patters with the expression of ER gene in HUSMCs and THP-1 cells, which may suggest a novel mechanism involved in atherogenesis by hyperhomocystine.

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