ISSN 1672-9145 **Acta Biochimica et Biophysica Sinica** 2007, **39**(1): 19–26 **CN** 31-1940/Q

Different Effects of Homocysteine and Oxidized Low Density Lipoprotein on Methylation Status in the Promoter Region of the Estrogen Receptor α Gene

Yushan HUANG[1], Kejun PENG[2], Juan SU[1], Yuping HUANG[3], Yizhou XU[1], and Shuren WANG[1]*

[1] *Department of Pathophysiology, West China School of Preclinic Medical Sciences & Forensic Medicine, Sichuan University, Chengdu 610041, China;*
[2] *Department of Laboratory Medicine, Chengdu Medical College, Chengdu 610500, China;*
[3] *Department of Biochemistry and Molecular Biology, Gannan Medical College, Ganzhou 341000, China*

Abstract We investigated the effects of homocysteine (Hcy) and oxidized low density lipoprotein (ox-LDL) on DNA methylation in the promoter region of the estrogen receptor α (ERα) gene, and its potential mechanism in the pathogenesis of atherosclerosis. Cultured smooth muscle cells (SMCs) of humans were treated by Hcy and ox-LDL with different concentrations for different periods of time. The DNA methylation status was assayed by nested methylation-specific polymerase chain reaction, the lipids that accumulated in the SMCs and foam cell formations were examined with Oil red O staining. The proliferation of SMCs was assayed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. The results showed that ox-LDL in moderate concentrations (10–40 mg/L) induced *de novo* methylation in the promoter region of the ERα gene of SMCs. However, high concentrations (50 mg/L) of ox-LDL, resulted in demethylation of ERα. The Hcy treatment resulted in *de novo* methylation in the promoter region of the ERα gene with a concentration- and treating time-dependent manner, and a dose-dependent promoting effect on SMC proliferation. These data indicated that the two risk factors for atherosclerosis had the function of inducing *de novo* methylation in the promoter region of the ERα gene of SMCs. However, high concentrations (50 mg/L) of ox-LDL induced demethylation, indicating that different risk factors of atherosclerosis with different potency might cause different aberrant methylation patterns in the promoter region of the ERα gene. The atherogenic mechanism of Hcy might involve the hypermethylation of the ERα gene, leading to the proliferation of SMCs in atherosclerotic lesions.

Key words homocysteine; oxidized low density lipoprotein; estrogen receptor α; DNA methylation; atherosclerosis

DNA methylation is an epigenetic process leading to the chemical modification of a genome [1]. The addition of the 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" to environmental factors. Accumulating evidence has shown aberrant DNA methylation patterns in various diseases, including cancer, certain X-linked genetic diseases [2,3], autoimmune diseases [4], aging [5], etc. Until now, the estrogen receptor α (ERα) gene is the only gene known to have aberrant hypermethylation in its promoter region in atherosclerosis development [6]. Homocysteine (Hcy) and oxidized low density lipoprotein (ox-LDL) are two established risk factors for atherosclerosis. So what are the effects of the two risk factors on the methylation pattern of ERα gene?

ERα gene is a potential growth suppressor gene [7]. *In vivo* and *in vitro* models of vascular disease have found estrogen to be protective against smooth muscle cell (SMC) proliferation and neointima formation [8]. Clinical evidence has also strongly suggested that estrogen replacement

Received: October 10, 2006 Accepted: November 27, 2006
This work was supported by the grants form the Specialized Research Fund for the Doctoral Program of Higher Education (No. 20050610050) and the Science and Technology plan project of the Department of Health of Jiangxi Province (No. 20062033)
*Corresponding author: Tel, 86-28-85501268; Fax, 86-28-85503204 ; E-mail, wangshuren1945@yahoo.com.cn

DOI: 10.1111/j.1745-7270.2007.00251.x

therapy in postmenopausal women might help prevent cardiovascular disease [9]. If the two important risk factors for atherosclerosis could exert a profound influence on the methylation pattern of the ERα gene, it would be most helpful in our understanding of the mechanisms of atherosclerosis development, and might even reveal some missing knowledge links between risk factors and atherosclerosis development. We therefore designed the current study to investigate the potential effects of Hcy and ox-LDL with various concentrations and treating times on the methylation pattern of the ERα gene, and their possible correlation with foam cell formation and SMC proliferation.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM)/F12 medium and RPMI 1640 were purchased from Gibco Life Technology (Burlington, Canada). Maleic dialdehyde (MDA) assay kit was purchased from the Jiancheng Bioengineering Institute (Nanjing, China). Homocysteine, oil red O, sodium bisulfite, hydroquinone and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were all obtained from Sigma-Aldrich (St. Louis, USA). Neonatal bovine serum (NBS) was from Sijiqing Biotechnologn Company (Hangzhou, China). Mouse anti-α-actin antibody was from Beijing Zhongshan Golden Bridge Biotechnogn Company (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 Company (Beijing, China). DNA mate and methylase AluI were from TaKaRa Biotechnology Company, Ltd. (Dalian, China).

Cell culture and treatment

Fresh human umbilical cords were obtained with informed consent from women with normal pregnancies undergoing abdominal delivery in the West China Second Hospital (Chengdu, China). 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 the explant technique. After removal of the endothelium and adventitia, the remaining tissue was cut into small pieces, planted on a tissue culture flask using Pasteur-pipette, and then bathed in DMEM/F12 medium supplemented with 20% NBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cultures were maintained at 37 ºC in a 5% CO_2 humid atmosphere. Monolayer confluent vein smooth muscle cells between 3–5 passages were used for all experiments. Cells were identified as vein smooth muscle cells by positive staining with SMC-specific α-actin antibody, a marker of SMCs.

Confluent (85%–95%) human umbilical smooth muscle cells (HUSMCs) were washed twice with phosphate-buffered saline (PBS) before the experiments. Then the SMCs were treated with 50, 100, 200, 500, and 1000 μM Hcy in serum-free DMEM/F12 medium for 24, 48, and 72 h, or with 10, 20, 30, 40, and 50 mg/L ox-LDL for 48 h in DMEM/F12 medium, respectively.

Preparation of nLDL and ox-LDL

Human native low density lipoprotein (nLDL) was prepared by a density gradient ultracentrifugation of plasma from a healthy blood donor with a density adjustment by sodium bromide [10], followed by dialysis against PBS at 4 ºC for 36 h to remove EDTA.

The nLDL was exposed to 10 mM $CuSO_4$ for 20 h at 37 ºC [11]. The resultant ox-LDL was then dialyzed against PBS with 100 μM EDTA at 4 ºC for 24 h, followed by concentration with macrogol 2000 (Sigma-Aldrich). Ox-LDL was then passed through a 0.22 μm millipore filter for sterilization. Identification of purity was carried out by 5% polyacrylamide gel electrophoresis.

The protein content of nLDL and ox-LDL was determined using the Coomassie Brilliant Blue G-250 method with albumin as the standard.

The oxidative extent of ox-LDL was monitored by measuring the thiobarbituric acid-reactive substance (TBARS) using the MDA assay kit. TBARS was calculated as micromole malondialdehyde per gram protein. The content of TBARS in the ox-LDL was 21 μmol/g.

Oil red O staining for foam cells

HUSMCs were planted in a coverslip pretreated by 0.1% polylysine in six-well plates. After 48 h of treatment by ox-LDL, the cells were washed three times with PBS, fixed in 2.5% glutaraldehyde for 3 h, dipped in 2.5% potassium dichromate for 16 h, and stained in 1% oil red O for 20 min to identify lipid droplets in the cytoplasm under a microscope. Cell nuclei were then re-stained in hematoxylin for 5 min. Foam cells were counted under a microscope. Commonly in foam cells the area of lipid droplets exceeded the area of the nucleus. Manual quantitative analysis of foam cells under a microscope was carried out by randomly pre-selected view fields to count the percentages of positive oil red O staining cells.

MTT assay for cell viability

Cell viability was determined by a colorimetric assay based on the ability of viable cells to metabolize MTT. MTT is a yellow tetrazolium salt that forms a blue formazan dye precipitate that can be extracted using an organic solvent when it is reduced by the mitochondria of metabolically active cells [12]. The HUSMCs were plated in 96 well plates at a density of 2×10^5 cells/well in DMEM/F12 medium with 20% NBS. After 48 h, the HUSMCs were washed twice with PBS, and then were treated with 50, 100, 200, 500 and 1000 μM Hcy in serum-free DMEM/F12 medium for 24 h, respectively. Three hours before the end of the cell incubation periods, the culture medium was added with 20 μl of MTT (5 g/L) to each culture well. After an additional 3 h of incubation at 37 ºC, the medium was removed and formazan crystals were dissolved in 0.2 ml dimethyl sulfoxide for 30 min at 37 ºC. The optical density (OD) of each well was measured at 560 nm using a microplate reader (Molecular Devices, Seattle, USA). The data are expressed as percentages of the control viability measured in untreated cells.

DNA extraction and sodium bisulfite treatment

DNA extraction and sodium bisulfite treatment were carried out as previously described [13]. Briefly, the cultured HUSMCs were collected by scraping with a cell scraper, and washed three times with PBS. Total DNA was extracted from cells using a total DNA extraction kit, according to the manufacturer's protocols.

Ten micrograms of DNA in 50 μl of Tris-EDTA Buffer were denatured with 5.5 μl of 3 M NaOH at 37 ºC for 10 min, followed by a 16 h treatment at 50 ºC after adding 30 μl 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 3 M NaOH, followed by ethanol precipitation with 33 μl of 3 M 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 65 ºC for 10 min. The DNA sample was immediately stored at –20 ºC until further use.

Nested methylation-specific-polymerase chain reaction

Nested methylation-specific-polymerase chain reaction (nMS-PCR) was used for the detection of methylation in the promoter regions of the ERα 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. Methylated cytosine residues are retained as cytosine at CpG sites, and are then used 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 carried out with the conventional PCR primers. Primers for the promoter region of the ERα gene were designed to include eight CPG dinucleotides that have been linked to the regulation of the ERα gene expression. The summary of the primers and product sizes of the nMS-PCR assays are shown in **Table 1**.

PCR products were gel purified with an agarose gel DNA fragment recovery kit according to the manufacturer's instructions and were sequenced by Invitrogen (Carlsbad, USA). To reduce mispriming and to increase efficiency, touchdown (TD) PCR was used in the amplification. Following hot start, samples were subjected to 20 cycles in a TD program (94 ºC for 45 s, annealing temperature for 45 s and 72 ºC for 45 s for 20 cycles, followed by a 1 ºC decrease of the annealing temperature every second cycle). After completion of the TD program, twenty cycles were subsequently run (94 ºC for 45 s, 45 ºC for 45 s and 72 ºC for 45 s), ending with a

Table 1 Sequence, location and characterization of the primers used in polymerase chain reaction (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		

ER-n, nested prime; ER-U, unmethylation primer; ER-M, methylation primer.

5 min extension at 72 ºC.

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

A HUSMC DNA sample without any treatment was used as the unmethylated control, and the methylated control DNA target was prepared through treating DNA with methylase AluI that methylates cytosine residues within all CpG dinucleotides *in vitro*.

Isolation of total RNA

Total RNA was extracted from the HUSMC treated with 200 μM Hcy in serum-free DMEM/F12 medium for 24, 48 and 72 h with BIOZOL reagent, according to the manufacturer's protocols. The purity of RNA samples was checked by measuring the absorbance of RNA at 260 and 280 nm and calculating A_{260}/A_{280}. RNA samples with A_{260}/A_{280}=1.8 were used for further studies. For checking the integrity of RNA samples, agarose formaldehyde gel was used. Samples with intact bands of 28S and 18S RNA were used for the semiquantitative RT-PCR.

Semiquantitative RT-PCR

Semiquantitative RT-PCR was carried out using a Boracker RT-PCR kit. Briefly, for the reverse transcription reaction, 3 μl of total RNA together with 1 μl of Oligo (dT)18 (50 pmol/μl) was denatured at 75 ºC for 5 min, then put on ice for at least 1 min. It was then added to 4 μl of 5×RT buffer, 2 μl of dNTP mixture (each 10 mM), 1 U of RNase inhibitor, and 1 μl of ReverTra Ace at a final volume of 20 μl. The reaction was allowed to proceed for 1 h at 42 ºC. After completion of the reaction, the enzyme was inactivated at 99 ºC for 5 min, then stored at –20 ºC until further use. Two and a half microliters of the total cDNA sample were amplified in a single-stage PCR using primers designed for the ERα and the housekeeping genes, human glyceraldehyde-3-phosphate dehydrogenase (G3PDH), with primers as follows: ERα forward, 5′-CCCTTGCTATGTTACTAAGCGTGAG-3′ (2387–2412); ERα reverse, 5′-TGCCATAGGAATACAAGAGGGTGCT-3′ (2652–2627); G3PDH forward, 5′-ACCACAGTCCATGCCATCAC-3′; G3PDH reverse, 5′-TCCACCACCCTGTTGCTGTA-3′.

Amplification of cDNA specific for the ERα gene was carried out using primers resulting in a PCR product of 266 bp, and amplification for G3PDH resulted in a PCR product of 450 bp. Each reaction mixture contained 0.25 mM dNTPs, 1 μl of primers (20 μM), 1.25 U Taq DNA polymerase and PCR buffer at a total volume of 25 μl. The following parameters were used for amplification: denaturation at 94 ºC for 45 s, annealing at 61 ºC for 45 s and extension at 72 ºC for 45 s. At the end of the desired cycles, the final incubation was carried out at 72 ºC for 5 min. After amplification of all samples for 30 cycles, 10 μl of PCR products were electrophoretically separated on 1% agarose gel. The quantification of RT-PCR products was carried out by measuring the densitometric analysis of agarose gels using an image analyzer (NIH image), then, the ratios (ERα/G3PDH) were calculated.

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 least significant difference test, and qualitative data were analyzed using a χ^2 test. Values less than 0.05 were considered significant.

Results

The effect of ox-LDL on foam cell formation

The SMC treated by ox-LDL for 48 h displayed a remarkable and dose-dependent lipid accumulation as shown in **Figs. 1** and **2**. The foam cells increased linearly with the increment of ox-LDL concentration, which was in accordance with the knowledge that ox-LDL is a risk factor for atherosclerosis.

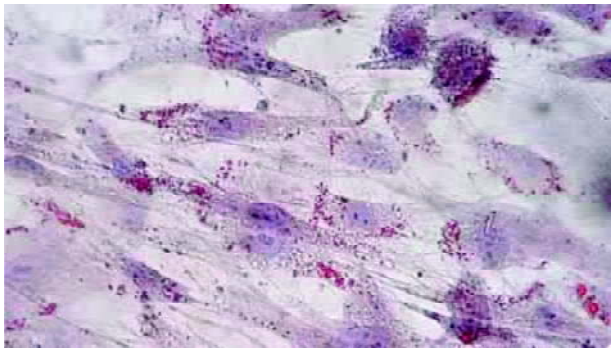

Fig. 1 Positive oil red O staining human umbilical smooth muscle cells (magnification, 400×)

The effect of Hcy on cell viability

Fig. 3 displayed an increasing proliferation of SMC along with the increment of Hcy concentration, which indicated

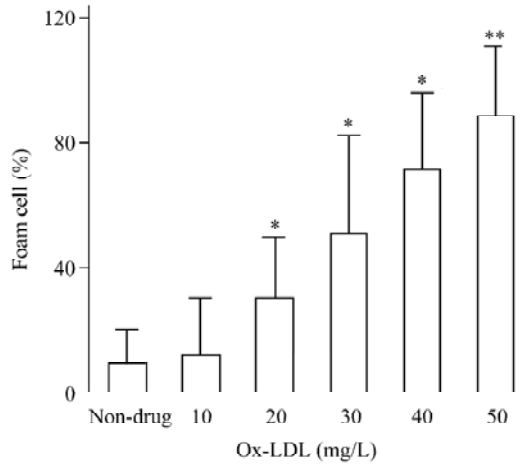

Fig. 2 The increase of percentage of foam cells along with the increment of oxidized low density lipoprotein (ox-LDL) concentration (*n*=6)

* *P*<0.05 and ** *P*<0.01 versus control (non-drug group).

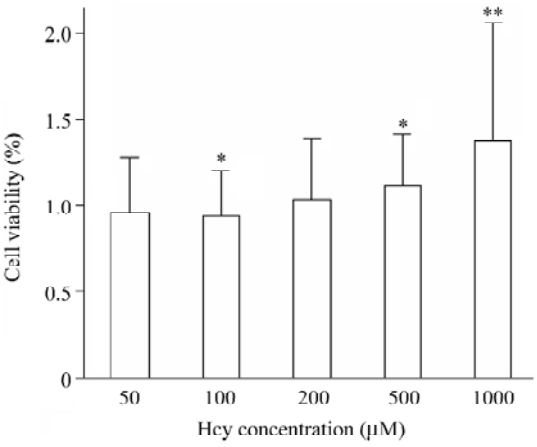

Fig. 3 Effect of homocysteine (Hcy) on cell viability: dose-dependent promoting effect of Hcy concentration on SMC proliferation

* *P*<0.05 and ** *P*<0.01 versus control (Hcy-untreated group).

a dose-dependent promoting effect of Hcy on SMC proliferation.

Alterations of methylation status of the ERα gene in HUSMCs treated by ox-LDL and Hcy with different concentrations and different treating times

Fig. 4 displayed the results of the methylation status of the ERα gene with different treatments. **Fig. 4(A)** illustrates the effect of ox-LDL on the methylation status of the ERα gene. DNA from normal HUSMC (without any treatment) amplified only with the unmethylated primers, which suggested that the promotion region of the ERα gene of SMC was unmethylation under normal conditions. The ox-LDL induced *de novo* methylation of the promotion region of the ERα gene in mild-moderate concentrations. All of the methylated primers and unmethylated primers produced evident PCR products in 10 mg/L–40 mg/L of ox-LDL. Ox-LDL in high concentrations (50 mg/L), however, showed a demethylation effect on the ERα gene. The PCR product with methylated primer was totally lost, possibly indicating that the effect of ox-LDL on *de novo* methylation of the ERα gene is not a function that is associated with the increase of ox-LDL concentration.

Fig. 4(B–D) illustrated the effect of Hcy with different concentrations and different treating times. The results exhibited significant dose-dependent and treating time-dependent *de novo* methylation in the promotion region of the 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 the 72-h treating group the unmethylated bands were totally lost, leaving only methylated bands.

The above results indicate that the two important risk factors of atherosclerosis could exert their effects on atherosclerosis development by inducing aberrant methylation in the promotion region of the ERα gene. This generally occurred in hypermethylation, which might interfere with expression, but the exerting behavior might vary with potency.

Alterations of ERα mRNA expression in HUSMCs with a different methylation status of the ERα gene

To determine whether the ERα promoter methylation status was associated with ERα mRNA expression levels, we used semiquantitative RT-PCR to examine ERα mRNA expression in HUSMCs treated with 200 μM Hcy for 24, 48 and 72 h (**Fig. 5**). From the above results, we found that along with the prolongation of treating time, the methylation degree was increased. We also found that along with the prolongation of treating time, the ERα mRNA expression was decreased.

Discussion

Atherosclerosis is a disease of large and medium-sized arteries and is characterized by lipid accumulation and SMC migration and proliferation [14,15]. Multiple mechanisms have been implicated in its pathogenesis. The earliest recognized gross lesion in atherogenesis is the fatty streak,

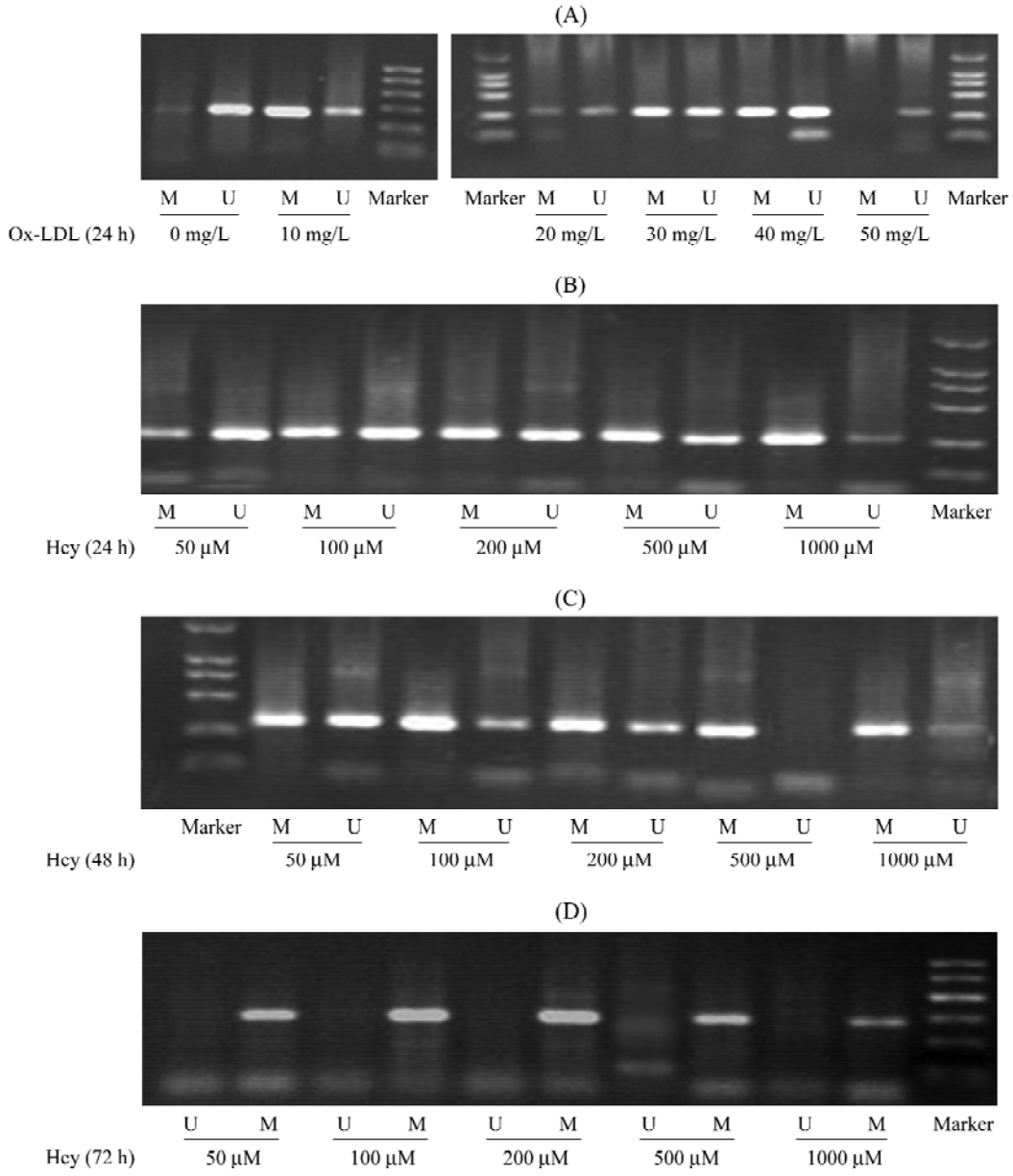

Fig. 4 The alteration of methylation status of ERα gene treated by oxidized low density lipoprotein (ox-LDL) and homocysteine (Hcy) with different concentration and different treating time

U, the unmethylated PCR band with primers; M, the methylated PCR band with primers.

characterized by an accumulation of cells loaded with cholesteryl esters (foam cells) just beneath the endothelium. Oxidized LDL has been implicated for playing a critical role in foam cell formation. The uptake of ox-LDL by monocyte/macrophage through scavenger receptors is the earliest and essential process in foam cell generation. Our data also show an obvious and dose-dependent promoting effect of ox-LDL on foam cell formation [16].

Ox-LDL is believed to exert diverse biological effects on atherosclerosis genesis. Sukhanov *et al.* used two cDNA microarray systems that contain a total of 35,932 unique genes to identify the genes differentially regulated by ox-LDL in human aortic smooth muscle cells (HASMC). They observed significant increases in RNA levels for 180 named genes and significant decreases for 192 named genes. They demonstrated that ox-LDL predominantly elevates the expression of genes involved in cell-cell interactions, membrane transport, oncogenesis, apoptosis,

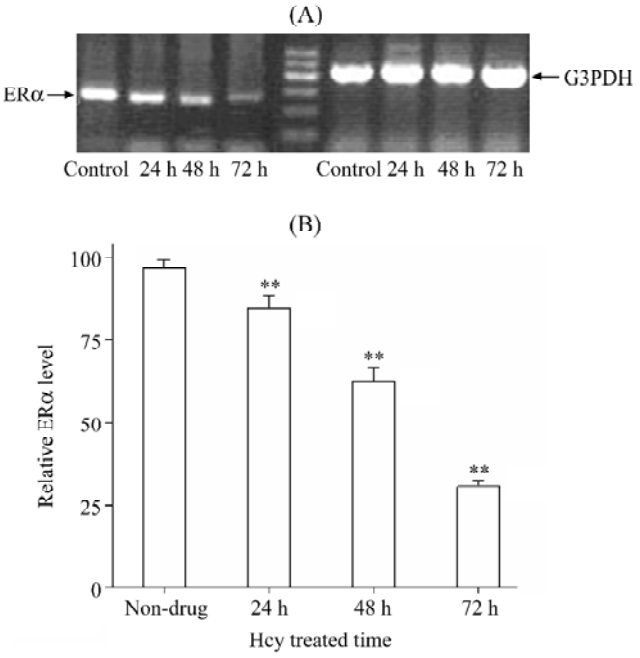

Fig. 5 Semiquantitative RT-PCR assessment of ERα gene expression in human umbilical smooth muscle cells treated by homocysteine (Hcy) with different treating time

** $P<0.01$ versus control (non-drug group).

and transcription and decreases the expression of genes responsible for protein and nucleic acid biosynthesis, lipid metabolism, and humoral responses [17].

Zaina *et al.* [18] recently reviewed the evidences proposing that lipids and lipoproteins can act as nuclear factors regulating chromatin structure and gene expression. The interaction between chromatin and small lipid molecules, such as cholesterol and lipid peroxidative products, have also been proved by increasing experimental evidences. And the association of hyperlipidemic lipoprotein profiles and aberrant DNA methylation patterns at early stages of atherosclerosis was also been proved in mice and in cultured human macrophages

ERα, upon activation by estrogen, regulates a variety of cellular activities, including the inhibition of cell proliferation. Such an anti-proliferative effect also involves the proliferation of SMCs [19]. To date, ERα is 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 [20]. Since the ox-LDL played an important role in foam cell formation and could modify chromatin structure to exert epigenetic regulations on gene expression, could the ox-LDL also play an atherogenic role by way of inducing aberrant methylation in the promoter sequence of the ERα gene? The present result shows that the promotion region of the ERα gene in HUSMCs displays *de novo* methylation upon the treatment of mild–moderate concentrations of ox-LDL. However, high concentrations of ox-LDL caused demethylation in the promotion region of the ERα gene. There is no dose-effect relationship between the ox-LDL concentration and the methylation level, but our data showed a clear dose-dependent promoting effect of ox-LDL on foam cell formation. The findings of this study can bring forward a tentative idea that the ox-LDL might not primarily play its pathogenic role in foam cell formation and atherogenesis by resulting in aberrant methylation in the promoter of the ERα gene.

Homocysteine, when its concentration is elevated in plasma, has been considered an independent risk factor for cardiovascular disease. The atherogenic mechanism of hyperhomocysteinemia can involve a variety of effects, including vascular endothelial dysfunction/injury, attenuation of NO-mediated vasodilatation, disturbance in the antithrombotic activities of the endothelium, oxidative stress, and activating nuclear factor-κB leading to recruitment of leukocytes and monocytes etc. [21,22]. Homocysteine is also intimately associated with S-adenosylmethionine, the methyl donor for more than 100 different transmethylation reactions, including DNA methylation [23,24]. So hyperhomocysteine can interfere with the epigenetic modification of a genome. Hiltunen *et al.* [25] have reported that a genomic hypomethylation occurred during atherogenesis in human, mouse and rabbit lesions and MTase was expressed in atherosclerotic lesions. Altered gene expression and cell proliferation in atherosclerotic lesions have some similar characteristics with certain solid tumors, which have shown genomic hypomethylation and hypermethylation 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 perhaps Hcy could exert its atherogenic effect by inducing abberant methylation patterns in the ERα gene.

In fact, the data in the present study demonstrate a dose-dependent *de novo* methylation in the promotion region of the ERα gene along with the increasing homocysteine concentration and treating time. We found that hypermethylation status of promoters could inhibit the ERα expression. Meanwhile the stimulated cell viability assay showed a dose-dependent promoting effect of Hcy on SMC proliferation. This result potentially suggests that the *de novo* methylation in the promotion region of the ERα gene,

induced by Hcy might involve the mechanism of SMC proliferation in atherogenesis.

In summary, our experimental results indicate that both ox-LDL and Hcy could cause aberrant methylation in the promotion region of the ERα gene, but the ox-LDL-induced aberrant methylation didn't exhibit any clear correlation with its effect of inducing foam cell formation. However, the Hcy-induced hypermethylation in the promotion region of the ERα gene showed similar dose-dependent patterns with the promoting effect on SMC proliferation, which might suggest a novel mechanism involved in atherogenesis by hyperhomocystine.

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Edited by
Minghua XU