Original Article

Homocysteine-mediated cholesterol efflux via ABCA1 and ACAT1 DNA methylation in THP-1 monocyte-derived foam cells

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Homocysteine (Hcy) has been recognized as a prevalent risk factor for cardiovascular events. Cholesterol-loaded foam cells are a central component of atherosclerotic lesions. ATP-binding cassette transporter A1 (ABCA1), which mediates the efflux of cellular cholesterol and phospholipids, is the rate-limiting step in lipid metabolism. Acyl-coenzyme A:cholesterol acyltransferase-1 (ACAT1) promotes accumulation of cholesterol ester in macrophages, thereby resulting in the foam cell formation, a hallmark of early stage in atherosclerosis. In this study, cultured monocyte-derived foam cells were incubated with clinical relevant concentrations of Hcy for 24 h. Both increased number of foam cells and accumulation of cholesterol were found, and the mRNA and protein expression levels of ABCA1 were decreased, while ACAT1 expression was increased in the presence of Hcy. Furthermore, the DNA methylation level of ABCA1 gene was increased whereas ACAT1 DNA methylation was decreased by using different concentrations of Hcy. Moreover, our results showed that DNA methyltransferase (DNMT) activity and DNA methyltransferase 1 (DNMT1) mRNA expression were increased by Hcy. It is indicated that DNA methylation has the function to regulate the expression of ABCA1 and ACAT1 via DNMT. In conclusion, these results suggest that ABCA1 and ACAT1 DNA methylation induced by Hcy may play a potential role in ABCA1 and ACAT1 expression and the accumulation of cholesterol in monocyte-derived foam cells.

Keywords homocysteine; ATP-binding cassette transporter A1; acyl-coenzyme A:cholesterol acyltransferase-1; DNA methylation; cholesterol efflux

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Introduction

Epidemiologic and case control studies have consistently indicated that moderate and mild elevation of plasma homocysteine (Hcy), an intermediate metabolite of methionine, is an important and independent risk factor in the development of atherosclerosis (AS) [1]. Hcy, a sulfur-containing amino acid derived from methionine, can be remethylated back to methionine as part of the methionine cycle. In this cycle, methionine is used for the synthesis of S-adenosylmethionine (SAM) which is converted into S-adenosylhomocysteine (SAH) and Hcy. SAM is the methyl donor for more than 100 different transmethylation reactions including DNA methylation which is catalyzed by DNA methyltransferases (DNMTs). DNA methylation leads to the suppression of gene expression when occurring in a regulatory region [2]. Hcy may be involved in the interference of DNA methylation [3], which might be an important mechanism of Hcy leading to AS. Significant genomic hypomethylation was found in lesions of ApoE knockout mice and neointima of balloon-denuded New Zealand white rabbit aortas [4] during the first replication of aortic SMCs in vitro, and hypomethylation occurred in some specific genes, such as extracellular superoxide dismutase and LDL receptor [5,6]. However, the molecular mechanisms contributing to Hcy-related foam cell formation are not fully known but may involve several pathways, including alterations in lipid metabolism and gene-specific changes in DNA methylation.

ATP-binding cassette transporter A1 (ABCA1), which is a member of a large family of ATP-binding cassette transporters and contains two ATP-binding domains and two six-helix transmembrane domains [7], is abundantly expressed in the peripheral macrophages among some tissues. ABCA1 is a key regulator of reverse cholesterol transport (RCT), the process by which accumulated cholesterol is transferred from the blood vessel walls to the hepatic tissue for excretion [8]. ABCA1 also mediates the transfer of cellular cholesterol and phospholipids to lipid-poor apolipoprotein A-I (apoA-I) to form nascent high-density lipoprotein (HDL) particles [9,10], so it is the rate-limiting step in generating plasma HDL [11,12]. HDL plays a role...
in transporting cholesterol from peripheral tissues to the liver, and the elevated level of HDL is associated with the decrease in the incidence of AS. Mutations in ABCA1 can cause low level of HDL and cholesterol accumulation in tissue macrophages and prevalent AS [13,14]. ABCA1 DNA methylation levels were found negatively correlated with circulating HDL-C, HDL2-phospholipid levels, and with a trend for association with HDL peak particle size. ABCA1 DNA methylation levels were also found associated with prior history of coronary artery disease (CAD). These results suggested that epigenetic changes within the ABCA1 gene promoter contribute to the interindividual variability in plasma HDL-C concentrations and are associated with CAD [15]. The dynamic equilibrium between free cholesterol (FC) and cholesterol ester (CE) in the cell is tightly catalyzed by the enzyme acyl-coenzyme A:cholesterol acyltransferase (ACAT) [16,17]. There are two isoforms of ACAT:ACAT1 and ACAT2. ACAT1, which is an integral membrane protein with multiple transmembrane domains, shows the major ACAT activity in macrophages for storing FC as lipid droplets to protect cells from the toxicity of intracellular FC [18,19]. Upregulation of ACAT1 expression impedes HDL-mediated RCT, which plays a critical role in the development of AS [20]. Preliminary studies of the role of ACAT in AS focused primarily on ACAT1 because of its expression in macrophages and its apparent role in foam cell formation within atherosclerotic plaques [21]. Studies show that leptin accelerates CE accumulation in human monocyte-derived macrophages by increasing ACAT1 expression, thereby suppressing cholesterol efflux [22]. Notably, the findings of Liao’s study indicated that hyperhomocysteinemia (HHcy) inhibits RCT by reducing circulating HDL via inhibiting apoA-I protein synthesis and enhancing HDL-C clearance [23]. An elevated level of Hcy leads to AS by affecting lipid metabolism and transport [24]. So it indicates that Hcy may be associated with the changes of ACAT1 methylation and expression in the formation of foam cells. In short, researches on the ABCA1, ACAT1, and Hcy, respectively, have been conclusive. Strong associations between lipid metabolism and Hcy have been observed in many retrospective and prospective studies [25], but the precise mechanisms of Hcy-inducing foam cell formation remain obscure up to date.

Therefore, we carried out this study both to determine whether Hcy-induced accumulation of cholesterol and influenced ABCA1, ACAT1 expression in THP-1 monocyte-derived foam cells and to explore the mediating mechanisms which DNA methylation was involved in.

**Materials and Methods**

**THP-1 cell culture and cell treatment**

Human THP-1 cells (West China School of Preclinical and Forensic Medicine, Sichuan University, China), a human monocytic leukemia cell line, were cultured in RPMI-1640 medium containing 15% (v/v) fetal calf serum in a humidified atmosphere of 5% CO2 at 37°C. Cells were initially differentiated into macrophages by addition of 5 μM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St Louis, USA) for 48 h, and then macrophages were transformed into foam cells by incubation with 50 mg/L ox-LDL (Sigma-Aldrich) in medium together with different concentrations of Hcy and its antagonists (vitamin B12, VB12, and folic acid, FA) for 24 h. In our experiment, cells exposed to different concentrations of Hcy (50, 100, 200, and 500 μM) were as Hcy group, incubated with Hcy (100 μM) in combination with FA (30 μM) and VB12 (30 μM) as treatment group, and treated only with ox-LDL as control group.

**Oil red O staining for foam cells**

The cultured THP-1 foam cells were washed with phosphate buffered saline (PBS) for three times, fixed in 10% formaldehyde for 10 min, dipped in 60% ethanol for 1 min, and stained in 1% oil red O (Sigma-Aldrich) for 30 min to identify lipid droplets in cytoplasm. Cell nuclei were then stained in hematoxylin for 5 min. All procedures were washed with distilled H2O. Foam cells derived from monocytes were observed and photographed. The semi-quantitative analysis of foam cells was evaluated by the percentage of positive oil red O staining cells [26].

**Analysis of cellular cholesterol accumulation and cholesterol efflux**

The cultured THP-1 human foam cells were collected by scraping with a rubber scraper, and washed three times with PBS. After resuspended in 0.5 ml PBS (0.1 M, pH 7.4), each sample was sonified for 1 min using the microtip of a sonifier. Samples (0.1 ml) were used for the measurement of cellular protein by Coomassie Brilliant Blue G-250 method, and the others were used for the measurement of cell-associated cholesterol and lipid peroxides.

For the determination of total cholesterol (TC), as described previously [27], 0.1 ml of sample was added to 0.9 ml of assay solution (0.1 U/ml cholesterol oxidase, 1 U/ml horseradish peroxidase, 0.01 U/ml CE hydrolase, 0.05% triton X-100, 1 mM sodium cholate, and 0.6 mg/ml phydroxyphenylacetic acid in 0.1 M PBS, pH 7.4) and incubated at 37°C for 1 h. Fluorescence was measured with a spectrofluorophotometer (excitation, 325 nm; emission, 415 nm). The concentrations of FC were determined using the enzymatic reaction end method with a FC kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the instructions. CE was determined by subtracting FC from TC.
Quantitative real-time polymerase chain reaction for ABCA1, ACAT1, and DNMT1

Total RNA was extracted from the cultured foam cells by using Trizol reagent (Invitrogen, Grand Island, USA). Premier 5 software (PREMIER Biosoft International, Palo Alto, USA) was used to design the primers. The primer nucleotide sequences of ABCA1 (NM_005502.2) were 5'-GTCCCTTTCTCCGATTA-3' (forward) and 5'-CTTGGTCACCTCAGCAT-3' (reverse). The primer nucleotide sequences of ACAT1 (NM_001130823.1) were 5'-CCAGAAAGAAATGGCACA-3' (forward) and 5'-TTCTACAGCCGTCACG-3' (reverse). The primer nucleotide sequences of DNMT1 (NM_001130823.1) were 5'-GGAGGCCACGCAAAGAGTA-3' (forward) and 5'-GGGAGACACCAGCAAAGTA-3' (reverse). In brief, RNA was reversely transcribed to cDNA by the use of the RevertAid first strand cDNA synthesis kit (MBI, Vilnius, Lithuania) according to the manufacturer's protocol. The cDNAs were amplified by SYBER Green PCR kit (MBI). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primer was 5'-AGAAGGCTGGGGCTCATTTG-3', and the reverse primer was 5'-AGGGGCCATCCACAGTC TTC-3'. The quantitative real-time polymerase chain reaction (PCR) was carried out by applying an FTC-3000 real-time PCR detection system (Funglyn, Toronto, Canada) with the program as follows: at 94°C for 10 min, 50 cycles at 94°C for 15 s, at the annealing temperature of 51°C for 30 s and 72°C for 30 s. The RNA level of each gene was acquired from the value of the threshold cycle (Ct) of the real-time PCR related to that of GAPDH through the formula \( \Delta Ct = Ct_{\text{GAPDH}} - Ct_{\text{gene}} \). Final results were expressed as N-fold differences in the target gene expression and was relative to the calibrator termed 'N_target', which were determined as follows: \( N_{\text{target}} = 2^{\Delta Ct_{\text{sample}} - \Delta Ct_{\text{calibrator}}} \), where \( \Delta Ct \) values of the calibrator and sample were determined by subtracting the Ct value of the target gene from the Ct value of GAPDH.

Western blot analysis for ABCA1 and ACAT1

Cultured foam cells were harvested by scraping with a rubber scraper. The proteins were extracted with protein extraction kit (KeyGen Biotech, Nanjing, China). The protein concentration in cellular supernatant was determined by the BCA assay. Equal amounts of proteins (typically 40 μg) were separated on sodium dodecyl sulphate and polyacrylamide gel electrophoresis (SDS-PAGE) gel, and electrophoretically transferred to PVDF membrane at 300 mA for 1 h at 4°C, and then blocked in 5% skimmed milk for 2 h at room temperature. The membrane was incubated with monoclonal anti-ABCA1 antibody or anti-ACAT1 antibody (1:200 dilution; Jackson ImmunoResearch, West Grove, USA) in 10 ml primary antibody dilution buffer with the gentle agitation on a platform shaker overnight at 4°C. The membrane was then washed 3 times for 10 min/time with PBS plus Tween-20 (PBST) and incubated with goat anti-rabbit HRP-IgG secondary antibody (Jackson Immuno-Research) in PBS at 1:2000 dilution for 2 h at room temperature. After washed again three times with PBST, the membrane was incubated with enhanced chemiluminescence (ECL) solution for 1 min at room temperature, and then the excess ECL solution was drained. The control could not be dried out, but instead, was wrapped in a plastic wrap and exposed to X-ray film. The control value was expressed as 100%. Relative values were normalized to that of β-actin using the formula (relative value = experimental densitometry value × 100/β-actin value).

Nested touchdown methylation-specific PCR analysis for ABCA1 and ACAT1 DNA methylation

We used Wizard Genomic DNA Purification Kit (Promega, Madison, USA) to extract DNA from foam cells, and determined the purity and concentration. ABCA1 and ACAT1 DNA methylation levels were analyzed by EZ DNA Methylation-Gold™ Kit (Zymo Research, Orange, USA) according to the manufacturer’s instructions. After the bisulfite modification of genomic DNA, unmethylated cytosine residues were converted into uracils and then subjected to further conventional DNA amplification. The online websites http://www.ncbi.nlm.nih.gov/ and http://www.urogene.org/methprimer/index.html were used to design ABCA1 and ACAT1 DNA methylation primers. Two sets of inner and outer primers were designed and synthesized based on DNA sequences of ABCA1 and ACAT1 as follows (Table 1).

In accordance with the specification requirements of DNA remodified sodium, the reaction mixture of 25 μl contained 4 μl modified DNA, 1 μl upstream and 1 μl downstream of each primer, 12.5 μl GoTaq Colorless Master Mix (2 ×) (Promega), and nuclease-free water. PCR was performed using the following steps in a thermal cycler: 94°C 5 min, 94°C 30 s, 61.8°C 30 s, 72°C 1 min, 30 cycles, decreasing 0.5°C every cycle, 94°C 30 s, 46.8°C 30 s, 72°C 1 min, 20 cycles, 72°C 7 min, then held at 4°C. The products (4 μl) of PCR amplification were resolved by agarose gels electrophoresis. The gel was analyzed by gel imaging analyzing instrument with optical density analysis of methylation changes and unmethylation changes.

Enzyme-linked immunoassay-like analysis for DNMT enzymes

Foam cells were scraped off with a rubber scraper, and washed in PBS for three times, then re-suspended in 0.5 ml PBS (pH > 7.4) and lysised by ultrasonic wave for 1 min. The activities of DNMTs were detected according to the instructions of the enzyme-linked immunoassay-like kit (Epigentek, New York, USA). The optical densities were
measured in the microplate reader, and then converted to the intracellular contents of DNMTs.

**Statistical analysis**
Results were expressed as mean ± SEM. The data were analyzed using one-way analysis of variance and additional analysis by using the Student-Newman-Keuls test for multiple comparisons within different groups or t test for between two groups. *P < 0.05 was considered significant.

**Results**

**Oil red O-stained foam cells**
The lipid droplets of cells were highlighted by means of Oil red O staining. In THP-1 monocyte-derived macrophages, the accumulation of cholesteryl esters resulted in the foam cell formation, a hallmark of early atherosclerotic lesions [28]. We reported previously that Hcy accelerated the development of foam cells in the presence of PMA and ox-LDL, and the foam cells treated with 100 μM Hcy were the best [29]. The monocytes were induced to macrophages with PMA for 48 h, and then co-cultured with ox-LDL and along with 50, 100, 200, 500 μM Hcy, and 100 μM Hcy + VB12 + FA (100 + VB12 + FA) for 24 h. A great number of foam cells were identified by oil red O staining [Fig. 1(A,B)], thus suggesting that Hcy induced the formation of foam cells.

**Cholesterol ester accumulation and cholesterol efflux in monocyte-derived foam cells**
The ratio of CE/TC > 50% is the mark of foam cell. The intracellular TC, FC and CE of foam cells were measured by enzymatic end point determination in order to observe the effects of Hcy on CE efflux. The protein content in

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**Table 1 Inner and outer primers of ABCA1 and ACAT1 DNA methylation analysis**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Tm (°C)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCA1 left outer primer: 5’-AGGTTTTTGAAAGAAATAAAGATAAG-3’</td>
<td>69.4</td>
<td>418</td>
</tr>
<tr>
<td>ABCA1 right outer primer: 5’-AATTCAATACCTCAACAAAAAACAC-3’</td>
<td>65.6</td>
<td>139</td>
</tr>
<tr>
<td>ABCA1 left M primer: 5’-AATTTCATTGTTTCTGTTGTC-3’</td>
<td>65.4</td>
<td>138</td>
</tr>
<tr>
<td>ABCA1 right U primer: 5’-ATATCTTAAAAATCCCAACTCCTACATC-3’</td>
<td>65.4</td>
<td>441</td>
</tr>
<tr>
<td>ACAT1 left outer primer: 5’-TATCTTAATCCCAACATCTCTACATC-3’</td>
<td>63.0</td>
<td>126</td>
</tr>
<tr>
<td>ACAT1 right M primer: 5’-CTACACCTCTACCTAATATTCA-3’</td>
<td>63.0</td>
<td>123</td>
</tr>
<tr>
<td>ACAT1 left U primer: 5’-TATCTTAATCCCAACATCTCTACATC-3’</td>
<td>63.0</td>
<td>123</td>
</tr>
</tbody>
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M, methylated; U, unmethylated.

Figure 1 Lipid droplets visualization and quantification in THP-1 foam cells by Oil red O staining (A) THP-1 monocyte-derived foam cell formation in the presence of 50 mg/l ox-LDL. The monocytic THP-1 cells were cultured for 2 days in RPMI-1640 medium containing 15% FBS and PMA (5 μM) to induce the differentiation into macrophages, and then incubated in RPMI-1640 medium containing 50 mg/l ox-LDL for 24 h. The cells were stained with Oil red O and counterstained with hematoxylin. The cells treated without Hcy as control. Original magnification: 1000×. (B) Percentage of positive oil red O-stained foam cells formed in the presence of 50 mg/L ox-LDL with treatment of various concentrations of Hcy and 30 μM VB12 + 30 μM FA. The extent of foam cell formation was divided into three grades ++, ++++, and +++++, each standing for the intracellular lipid droplets occupied <1/3, 1/3–2/3, or >2/3 in cytoplasm, respectively. The percentage of foam cells in ++ and +++ grades was calculated and shown. *P < 0.05, **P < 0.01 vs. control group; ##P < 0.01 vs. 100 μM Hcy group.
ABCA1, ACAT1 mRNA, and protein expression analysis

To understand whether Hcy affects regulation of ABCA1 and ACAT1, we also examined the mRNA and protein expression levels of ABCA1 and ACAT1 in the presence of Hcy. The data showed that the mean values of expression of ABCA1 mRNA in the experimental groups (50, 100, 200, 500 μM Hcy and 100 + VB12 + FA) were, respectively, 63.08%, 72.32%, 76.77%, 62.13%, 51.30%, and 66.29%, respectively. All of them were more than 50%. The levels of CE/TC gradually increased and the maximum level was at 100 μM Hcy compared with the control group. Additionally, this increase was suppressed by FA and VB12 to the level similar to that of foam cells.

Homocysteine-induced ABCA1 and ACAT1 DNA methylation changes analysis

DNA methylation plays an important role in regulating gene expression [30]. We explored the effects of Hcy on the DNA methylation status of ABCA1 and ACAT1 promoter regions of THP-1 foam cells via nested touchdown methyl-specific PCR. The results showed that the increase of Hcy concentrations from 50 to 100 μM and further to 500 μM led to statistically significant differences (Fig. 4) compared with control group in ABCA1 and ACAT1 promoter region DNA methylation levels, in which the methylation changes of 100 μM group was the most obvious. It was found that in experimental groups (50, 100, 200, 500 μM Hcy), the levels of ABCA1 DNA methylation increased significantly by 38.28%, 55.98%, 47.12%, and 37.93%, respectively, whereas the levels of ACAT1 DNA methylation in the 100 μM Hcy group decreased significantly by 8.32% compared with the control. The level of ABCA1 DNA methylation in 100 + VB12 + FA group significantly decreased by 28.34% compared with 100 μM Hcy (P < 0.01), while the level of ACAT1 DNA methylation in 100 + VB12 + FA group significantly increased by 14.95% compared with 100 μM Hcy group (Fig. 4). That indicated Hcy could increase the level of ABCA1 promoter region DNA methylation, whereas Hcy could decrease ACAT1 promoter region DNA methylation level. Meanwhile, in 100 + VB12 + FA group, the DNA methylation levels of ABCA1 and ACAT1 reverted back to the levels observed in the control group (Fig. 4).

DNMTs activity and DNMT1 mRNA expression analysis

Establishment and maintenance of methylation patterns are mediated by a family of enzymes called DNMTs, which

![Figure 2 Intracellular cholesterol efflux in THP-1 foam cells](image-url)

Monocytes were initially differentiated into macrophages by addition of 5 μM PMA for 48 h, and then were transformed into foam cells by incubation with 50 mg/l ox-LDL together with different concentrations of Hcy or 100 μM Hcy + 50 μM VB12 + 30 μM folic acid for 24 h. TC and FC were measured by enzymatic end point determination, and CE was calculated by subtracting FC from TC. *P < 0.05, **P < 0.01 vs. control group; ***P < 0.01 vs. 100 μM Hcy group.
contain three active enzymes: DNMT1, DNMT3a, and DNMT3b in the human genome [31]. The DNMT activities of foam cells treated with different concentrations of Hcy were shown in Fig. 5(A). The results indicated that the DNMT activities were increased by Hcy, and the highest value was at the 50 μM Hcy. Meanwhile, the DNMT activity of 100 μM Hcy group was also significantly increased compared with the control group.

Figure 3 ABCA1 and ACAT1 mRNA and protein expression analysis of THP-1 foam cells. (A,B) Relative mRNA expression levels of ABCA1 (A) and ACAT1 (B). ABCA1 and ACAT1 genes RNA levels were acquired from the values of the threshold cycle (Ct) of the real-time PCR as related to that of GAPDH through the formula $\Delta \text{Ct} = \text{Ct(ATTG)} - \text{Ct(sample)}$. Final results, expressed as N-fold differences in ABCA1 and ACAT1 genes expressions relative to the calibrator, termed $N_{\text{gene}}$, were determined as $N_{\text{gene}} = 2^{\Delta \text{Ct(sample)} - \Delta \text{Ct(calibrator)}}$. The data were expressed as percentage of mean ± SEM. (C,D) Effects of Hcy on ABCA1, ACAT1 protein levels in THP-1 monocyte-derived foam cells. Foam cells were incubated in the absence or presence of Hcy at various concentrations. The immunoblots were analyzed by densitometry. The cell-lysated protein samples were freshly prepared from THP-1-derived foam cells, and separated on 12% SDS-PAGE (40 μg proteins/lane), followed by transferring to the PVDF membranes. The transferred-membranes were respectively incubated with the specific anti-ABCA1 or anti-ACAT1 antibody. The control value was expressed as 100%. Relative values were normalized to that of β-actin through the formula (relative value = experiment densitometry value $\times$ 100/β-actin value). The experiments were repeated more than three times with similar results. *$P < 0.05$, **$P < 0.01$ vs. control group; $\#P < 0.05$, $\##P < 0.01$ vs. 100 μM Hcy group.

Figure 4 Effects of Hcy on ABCA1 and ACAT1 DNA methylation in THP-1 foam cells. Nested touchdown methylation-specific PCR was performed after bisulfite modification of DNA from THP-1 foam cells treated with or without Hcy for 24 h. (A,C) Representative bands. U indicates unmethylated ABCA1 or ACAT1 PCR products. M indicates methylated PCR products. (B,D) The promoter methylation values of ABCA1 and ACAT1 PCR products by densitometry based on biological triplicates. *$P < 0.05$, **$P < 0.01$ vs. control group; $\#P < 0.05$, $\##P < 0.01$ vs. 100 μM Hcy group.
The DNMT1 is well known for its function as a major maintenance DNMT enzyme [32]. Therefore, in our studies, we detected the mRNA expression of DNMT1. DNMT1 mRNA expression levels in the experimental groups were increased compared with the control group, and the highest was at the 100 μM Hcy. But the increasing of Hcy concentrations did not cause a dose-dependent increase in DNMT1 mRNA expression [Fig. 5(B)].

**Discussion**

AS is a complex disease characterized by accumulation of lipids, fibrous materials, cell debris, and minerals in the intima of arteries. A growing body of evidence has documented the role of Hcy as an independent vascular risk factor in AS. However, the mechanisms of Hcy-caused AS remain elusive.

Cholesterol accumulation within atherosclerotic plaque occurs when cholesterol influx into the arterial wall exceeds cholesterol efflux [33]. We detected the intracellular lipids, including TC, FC, and CE as well as the percent of foam cells. In our results, PMA-activated THP-1 monocytes showed a significant increase in foam cell transformation when cultured with ox-LDL and Hcy compared with control, which indicated that the model of foam cells was constructed successfully. Moreover, Hcy interfered with lipid metabolism and speeded up the accumulation of cholesterol, indicating Hcy might be an important factor involved in the accumulation of cholesterol and the formation of foam cells.

In macrophages, the effects of ABCA1 on cholesterol efflux may be critical for protecting the artery wall from atherosclerotic lesion formation [34]. ACAT1 is expressed at high levels by macrophage-derived foam cells in atherosclerotic lesions [35], and ACAT1 influences the efflux of both cellular and lipoprotein-derived cholesterol [36]. Our results indicated that mRNA and protein expression of ABCA1 was decreased whereas ACAT1 mRNA and protein expression was increased by Hcy in foam cells derived from the monocytes. These results indicated that Hcy caused the changes of ABCA1 and ACAT1 expression, which provided evidence that ABCA1 and ACAT1 genes were important genes responsible for Hcy-induced atherogenesis.

Increasing evidence indicated that Hcy may be involved in disturbing the expression of AS-related genes through the interference of DNA epigenetic phenotype modification [29]. DNA methylation is a major epigenetic modification of the genome that has the potential to silence gene expression. DNA methylation is a form of epigenetic gene regulation that together with altered binding profile of transcription factors commonly leads to suppression of gene expression when occurring in a regulatory region. ABCA1 and ACAT1 play a key role in intracellular lipid metabolism, influencing the processes such as intracellular routing of endocytosed remnant lipoproteins, and cholesterol efflux to HDL [37, 38]. However, so far, there has been no evidence to elucidate the relations between DNA epigenetic phenotype modification of ABCA1, ACAT1 and the formation of foam cells. Our data showed that ABCA1 promoter was hypermethylated while ACAT1 promoter was hypomethylated after exposed to Hcy. The results indicated a causal relationship between the expression of ABCA1 and ACAT1 and the DNA methylation changes, and then ascertained the mechanism of Hcy-induced AS. In addition, DNMT controls methylation in both suppressor and promoter genes, and other circumstances may be determining factors for or against AS in association with the CpG-island methylation. The data from our study displayed that the transcriptional level of DNMT1 and the activity of DNMTs increased in THP-1 monocyte-derived foam cells after incubation with Hcy, which might be associated with the DNA methylation changes of ABCA1 and ACAT1, and a potential yet plausible explanation was the compensatory reaction of the methylation mechanisms against Hcy-induced genomic DNA (gDNA) hypomethylation. These findings implied two potential genic events occurring in foam cells. It has been shown that hypermethylation resulted from increased...
DNMT gene expression is an independent event found in a number of haematological malignancies [39]; the presumed mechanism is silencing of genes such as ABCA1 in our study. Paradoxically, increased DNMT activity has been related to DNA hypomethylation just as ACAT1 in our study, a fact that is explained by decreased levels of the methyl donor SAM in some tumor cell types [40], and the decline of SAM might be resulted from the excessive consumption due to the increased activity of DNMT [41]. It has been reported that HHcy could lead to hypomethylation of gDNA, also explaining the hypomethylation activity of ACAT1 DNA promoter region. In order to elucidate ABCA1 hypermethylation and ACAT1 hypomethylation, further study involving microRNA regulation is needed to characterize gene-specific hypermethylation and hypomethylation in the presence of Hcy.

The dosage of Hcy used in this study is clinically relevant from moderate (Hcy concentration of 50 μM, which is found in up to 40% of patients with myocardial infarction, stroke, or venous thrombosis) to severe hyperhomocysteinemia. The impacts of various concentrations of Hcy on the one-carbon methyl-group transferring metabolism, however, did not show dose-dependent manner relationship. This phenomenon suggested that the varied detrimental effects of Hcy could be attributed to different concentrations via different mechanisms, in mild and moderate hyperhomocysteinemia. Hcy may primarily influence the epigenetic regulation of gene expression through the interference of methyl group transferring metabolism while at much higher Hcy concentration, the essential impacts may be more directly injurious via oxidative stress, pro-apoptosis, inflammation etc.

VB12 and FA also play a key role in one-carbon metabolism and DNA methylation because they influence the supply of methyl groups and consequently the biochemical pathways of methylation processes [42, 43]. From our results above, we found that when VB12 and FA were supplemented to the 100 μM Hcy, the expression and DNA methylation levels of ABCA1 and ACAT1 reverted back to the levels observed in the control group. Our findings indicate that FA and VB12 may change the levels of ABCA1 and ACAT1 DNA methylation.

Finally, the findings of our study uncovered the methylation changes induced by Hcy in ABCA1 and ACAT1. It is possible that alterations in ABCA1 and ACAT1 methylation may play an important role in atherogenesis, though it did not prove a direct relationship among ABCA1 hypermethylation, ACAT1 hypomethylation and atherogenesis. As we know, the induction of ABCA1 gene hypermethylation and ACAT1 hypomethylation via Hcy still remain unrevealed and unreported in the field of their related mechanisms. Therefore, in addition to the important statistics and findings, this study has also revealed the novel and attractive role of Hcy in the pathogenesis of human cardiovascular disease.

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