FABP4-mediated homocysteine-induced cholesterol accumulation in THP-1 monocyte-derived macrophages and the potential epigenetic mechanism

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Abstract. Hyperhomocysteinemia (HHcy) is an independent risk factor for the development of atherosclerosis (AS), according to overwhelming number of clinical and epidemiological studies. However, the underlying pathogenic molecular mechanisms by which HHcy promotes AS remain to be fully elucidated. Fatty acid binding protein 4 (FABP4) has been shown to be important in macrophage cholesterol trafficking. The objective of the present study was to determine whether homocysteine (Hcy) accelerates AS through regulating FABP4, and then mediates cholesterol accumulation in macrophages. Hcy concentrations of 0, 50, 100, 200 and 500 µM, and 100 µM Hcy+30 µM vitamin B12 (VB12)+30 µM folic acid (FA) were respectively added to cultured THP-1 monocyte-derived macrophages for 24 h. The levels of FABP4, which acts as a key factor connecting cellular lipid accumulation to inflammation, were determined using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analyses in the macrophages. The present study used a nested touchdown methylation-specific PCR assay to detect the DNA methylation status of the FABP4 promoter region. In addition, the FABP4 gene fragment was inserted into the cloning vector, pcDNA3.1-EGFP, to construct the recombinant plasmid, pcDNA3.1-EGFP/FABP4, which was identified using restriction endonuclease digestion analysis and DNA sequencing. The pcDNA3.1-EGFP/FABP4 expression plasmid was transfected into THP-1 monocyte-derived macrophages, mediated by liposome reagent, following which the expression levels of FABP4 were detected using RT-qPCR and western blot analyses. The present study also determined the intracellular accumulation of total cholesterol in the macrophages. The results indicated that Hcy decreased the levels of FABP4 promoter methylation, but increased the mRNA and protein expression levels of FABP4 in the macrophages, compared with the control group (0 µM Hcy). However, no dose-dependent changes were observed with increasing concentrations of Hcy. The recombinant fluorescent eukaryotic expression vector, pcDNA3.1-EGFP/FABP4, was successfully constructed and effectively expressed in the THP-1 macrophages. The results also showed that FABP4 accelerated the accumulation of cholesterol in the macrophages. Taken together, the results of the present study suggested that FABP4 DNA hypomethylation induced by Hcy may be involved in the overexpression of FABP4, thereby inducing cholesterol accumulation in macrophages.

Introduction

Fatty acid-binding protein 4 (FABP4), also referred to as aP2 and adipocyte FABP, is expressed at high levels in adipocytes and macrophages, and is a cytosolic lipid chaperone, which regulates cellular lipid metabolism and the reception of lipid signals (1), which are actively involved in atherogenic processes. FABP4-deficiency has been shown to protect against atherosclerosis (AS) in apolipoprotein E-deficient mice, and FABP4 knockout animal models show reduced lipolysis, which offers protection from the development of AS (2). Investigations in bone marrow transplantation have also demonstrated that this atheroprotective effect of FABP4 is predominantly associated with its actions in macrophages (3). Thus, it is clear that the involvement of FABP4 is significant.
in local macrophage responses in atherogenesis, however, the precise cellular and molecular mechanisms underlying these phenomena remain to be fully elucidated. Previous studies have reported the presence of FABP4 in macrophages, and have shown that the expression of FABP4 can be induced by oxidized low density lipoprotein (oxLDL), peroxisome proliferator-activated receptor γ (PPARγ) agonists and the differentiation of monocytes to macrophages (4-6).

Homocysteine (Hcy) is a homologue of the amino acid, cysteine, differing by an additional methylene bridge (-CH2-). Accumulating evidence has suggested that hyperhomocysteinemia (HHcy) is an independent risk factor of AS (7). However, the mechanisms responsible, which may be multifactorial, remain to be fully elucidated (8). Increasing evidence has indicated that Hcy may be involved in the disturbance of the expression of AS-associated genes through the interference of DNA methylation (9). The metabolism of Hcy is specifically involved in the methionine cycle for transmethylation reactions, therefore, its elevation has been implicated in the interference of DNA methylation modification and the epigenetic regulation of genes (10).

DNA methylation, the addition of a methyl group to form 5-methylcytosine in the context of a CpG dinucleotide via methyltransferase, refers to a form of epigenetic modification. It has been demonstrated that aberrant DNA methylation is involved in Hcy-associated pathology, which exhibits global hypomethylation, however, hypermethylation occurs in certain genes. DNA hypermethylation leads to gene silencing, whereas hypomethylation results in gene activation in the regulatory region, together with altered binding profiles of transcription factors (11). Chronic human diseases, including AS, are either caused or affected by abnormal DNA methylation (12). Previous compelling evidence has indicated that Hcy epigenetically regulates certain specific targets, including 15-lipoxygenase, extracellular superoxide dismutase, estrogen receptor α, cyclin A and PPARs by inhibiting DNA methylation, which in turn favors early AS (13,14). Therefore, the interference of DNA methylation may be an important mechanism of Hcy-induced AS. However, whether Hcy can affect the DNA methylation and expression of FABP4 in macrophages, and further regulate cellular lipid metabolism remains to be elucidated.

Therefore, the present study hypothesized that Hcy, at a relevant concentration, affects the level of FABP4 DNA methylation and accelerates the development of AS through the accumulation of cholesterol in macrophages. Investigation to confirm this hypothesis may elucidate a potential mechanism against AS, in which epigenetic gene silencing is a feature.

Materials and methods

THP-1 cell culture and cell treatment. Human monocytic leukemia THP-1 cells (West China School of Preclinical and Forensic Medicine, Sichuan University, Sichuan, China) were cultured in RPMI 1640 medium supplemented with 15% (v/v) heat-inactivated fetal bovine serum (GE Healthcare Life Sciences, Logan, UT, USA) in a humidified atmosphere of 5% CO2 at 37°C. The monocytes were initially differentiated into macrophages by the addition of 500 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St. Louis, MO, USA) for 48 h, following which the macrophages were incubated (37°C, 5% CO2) in medium with different concentrations of Hcy and its antagonist for 24 h. In the present study, the cells were divided into five groups: Cells (2×10^6) were exposed to different concentrations of Hcy (50, 100, 200 and 500 μM), as the Hcy group, others were incubated with Hcy (100 μM) and folic acid (FA; 30 μM) and vitamin B12 (VB12: 30 μM), as the treatment group, and the remainder were treated without Hcy, as a control group. The cells in all groups were cultured for another 24 h, and all cells were used for subsequent analysis.

Construction of the pcDNA3.1-EGFP/FABP4 recombinant plasmid and its transfection into THP-1 monocyte-derived macrophages. The human FABP4 gene was amplified from the cDNA of THP-1 macrophages. The human FABP4 gene and the pcDNA3.1-EGFP vector were double-digested using EcoRI and HindIII restriction endonucleases (Takara Bio Inc., Otsu, Japan), and the FABP4 gene fragment was then inserted into the pcDNA3.1-EGFP cloning vector to construct the recombinant plasmid, pcDNA3.1-EGFP/FABP4. The recombinant pcDNA3.1-EGFP/FABP4 plasmid was identified using restriction endonuclease digestion analysis and DNA sequencing. According to the manufacturer’s protocol, the THP-1 macrophages grown in 6-well plates were transfected with the recombinant plasmid (pcDNA3.1-EGFP/FABP4) and empty plasmid (pcDNA3.1-EGFP), mediated by liposome reagent (Lipofectamine 2000; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) as the recombinant plasmid group and the empty plasmid control group, respectively. The untransfected group was set as the normal control group. The levels of green fluorescence in the cells were observed using fluorescence microscopy, and the expression levels of FABP4 were detected using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analyses.

RT-qPCR for the mRNA expression of FABP4. Total RNA was extracted from the cultured macrophages using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), following which the RNA was reverse transcribed into cDNA using a RevertAid first strand cDNA synthesis kit (MBI Fermentas, Inc., Vilnius, Lithuania), according to the manufacturer's protocol. The cDNAs (2 μl) were amplified using a SYBR Green PCR kit (MBI Fermentas, Inc.). Primer Premier 5 software (Premier Biosoft, Palo Alto, CA, USA) was used to design the primers. The primer nucleotide sequences of FABP4 (NM_001442.2; https://www.ncbi.nlm.nih.gov/nuccore/NM_001442.2) were as follows: Forward 5’-TACTGAGATTTCCCTTACTGGGC-3’ and reverse 5’-GCTCTCTCATAACTCTCGTGGAAG-3’, with a length of 372 bp. The gene expression of FABP4 was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5’-AGAAGGCTGGGGCTCATTTG-3’ and reverse 5’-AGGGGCCCCATCCACAGTCTTC-3’. The RT-qPCR reaction was performed using an FTC-3000 real-time PCR detection system (Funglyn, Toronto, Canada), according to the manufacturer’s protocol, as follows: 94°C for 10 min, 50 cycles at 94°C for 15 sec, at the annealing temperature of 53°C for 30 sec and 72°C for 30 sec. The RNA level of the gene was acquired from the value of the quantification cycle (Cq) of the RT-qPCR associated with that of GAPDH using the following formula: ΔCq = Cq_GAPDH - Cq_gene.
Table I. Inner and outer primers used for FABP4 DNA methylation analysis.

<table>
<thead>
<tr>
<th>FABP4</th>
<th>Primer sequence (5‘→3’)</th>
<th>Temperature (˚C)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer</td>
<td>Forward: ATTTTATTAGGAGGAAGGAAAAAA</td>
<td>71.1</td>
<td>414</td>
</tr>
<tr>
<td></td>
<td>Reverse: TCACATCTCAAAATCTCAAAACTAACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylated</td>
<td>Forward: AAGTTGGAAGTTTTTTTTGTAAACG</td>
<td>69.3</td>
<td>137</td>
</tr>
<tr>
<td></td>
<td>Reverse: CTTTGACCTATATTTACTTTCTTGCAGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unmethylated</td>
<td>Forward: AGTTGGAAGTTTTTTTTGTATGGG</td>
<td>69.2</td>
<td>134</td>
</tr>
<tr>
<td></td>
<td>Reverse: TTACCTATATTTACTTTCTTTCAAA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Western blot analysis for FABP4. The cultured macrophages were harvested by scraping with a plastic scraper and washing with phosphate-buffered saline (PBS). The protein was extracted using a protein extraction kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China), and protein concentration was determined using a Bicinchoninic Acid Protein Assay kit (Nanjing KeyGen Biotech Co., Ltd.). Equal quantities of protein (80 µg) and a known molecular weight marker were loaded onto 12% sodium dodecyl sulfate-polyacrylamide gels (Nanjing KeyGen Biotech Co., Ltd.) and were transferred onto a polyvinylidene fluoride membrane (EMD Millipore, Boston, MA, USA) by electrophoresis at 300 mA for 50 min at 4˚C. The membrane was then blocked in 10 ml 5% skimmed milk for 2 h at room temperature with gentle agitation on a platform shaker. The blocked membranes were respectively incubated with a rabbit monoclonal anti-FABP4 antibody (cat. no. MABS172; Abcam, Cambridge, MA, USA) in PBS at 1:2,000 dilution; a goat anti-rabbit horseradish peroxidase- IgG (cat. no. ab6721; Abcam, Cambridge, MA, USA) in PBS at 1:2,000 dilution; and β-actin antibody (cat. no. TA-09; 1:2,000 dilution; ZSGB-BIQ, Beijing, China) in 10 ml primary antibody dilution buffer overnight at 4˚C. The membrane was then washed three times for 10 min/wash with PBS-0.1% Tween-20 (PBST) and incubated with secondary antibody (cat. no. ab6721; goat anti-rabbit horseradish peroxidase-IgG; Abcam, Cambridge, MA, USA) in PBST at 1:2,000 dilution for 2 h at room temperature. Following being washed three times with PBST, the FABP4 on the membrane was incubated with enhanced chemiluminescence (ECL; Elabscience Biotechnology Co., Ltd., Wuhan, China) for 1 min at room temperature, following which the excess ECL solution was drained, and the membrane was wrapped in a plastic wrap and exposed to X-ray film (Kodak, Shanghai, China). The densities of the bands were quantified and the relative values were normalized to that of β-actin using the following formula: Associated value = experimental densitometry value / β-actin value.

Nested touchdown methylation specific-PCR (mMSP) analysis of FABP4 DNA methylation. Genomic DNA from the macrophages was isolated using a Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA) and modified using an EZ DNA Methylation-Gold™ kit (Zymo Research Corp., Orange, CA, USA), following which it was used as a template for mMPC. Following bisulfite modification of the genomic DNA, unmethylated cytosine residues were converted into uracil. The FABP4 DNA methylation primers for MSP amplification were designed using the MethPrimer bioinformatics program (http://www.urogene.org/methprimer/index.html). The inner and outer primers were designed and synthesized based on the DNA sequences of FABP4 (Table I).

To reduce mispriming and to increase efficiency, mMSP was used for amplification, which comprised a two-step PCR amplification procedure following the standard sodium bisulfite DNA modification. In accordance with the specification requirements of DNA remodified sodium, 4 µl of modified DNA was obtained, 1 µl upstream and 1 µl downstream of each primer, using Go Taq Colorless Master mix (x2; 12.5 µl; Promega), with nuclease-free water added to 25 µl. The samples were subjected to the following steps in a thermal cycler: 94˚C for 5 min, 94˚C for 30 sec, 74˚C for 30 sec and 72˚C for 1 min, for 30 cycles, decreasing 0.5˚C every cycle, 94˚C for 30 sec, for 59˚C 30 sec and 72˚C for 1 min, 20 cycles, and 72˚C for 7 min. The PCR products were separated by electrophoresis on 2% agarose gel. The bands were visualized under ultraviolet illumination and calculated using the following formula: Methylation (%) = methylation / (methylation + unmethylation) x 100%. Each sample was examined using mMSP analysis three times.

Assessment of intracellular total cholesterol (TC). The macrophages were scraped off the culture plate using a rubber scraper and washed in PBS three times, following which they were re-suspended in 0.5 ml PBS (pH 7.4) and lysed by ultrasound for 1 min. The TC was determined, according to the manufacturer's protocol of the Total cholesterol kit-CHO (Beijing BHKT Clinical Reagent Co., Ltd, Beijing, China). TC concentrations were expressed as mM/l.

Statistical analysis. Experiments were performed at least three times and the results are expressed as the mean ± standard error of the mean. The data were analyzed using one-way analysis of variance, and additional analysis was performed using the Student-Newman-Keuls test for multiple comparisons within different groups. GraphPad Prism 5.01 software was used for analysis (version 5.01, GraphPad Software, Inc.,
La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Formation of macrophages derived from activated THP-1 monocytes. The THP-1 monocytes were observed to grow in suspension and not adhere to the plastic surfaces of the culture flasks (Fig. 1A). For the induction of terminal differentiation into macrophages, the THP-1 cells were cultured in the presence of 500 nM PMA for 2 days, and then co-cultured with 50, 100, 200 and 500 mM Hcy or 100 mM Hcy+VB12+FA for 24 h. Following 72 h of culture, the cells had adhered and spread across the bottom of the dish, and had the morphological characteristics of macrophages (Fig. 1B).

Hcy-induced upregulation of the mRNA and protein expression levels of FABP4. To investigate whether the regulation of FABP4 occurred in the context of Hcy, the present study examined the mRNA and protein expression levels of FABP4 using RT-qPCR and western blot analyses in macrophages exposed to Hcy for 24 h. The data demonstrated that the expression of FABP4 was substantially upregulated following incubation with Hcy for 24 h at various concentrations (between 50 and 500 µM), and the mean mRNA expression levels of FABP4 in the experimental groups (50, 100, 200 and 500 mM Hcy, and 100+VB12+FA) were 10.84, 11.46, 5.89, 3.45 and 1.41 times higher, compared with that in the control group, respectively (Fig. 2A). The highest level of expression was observed at the concentration of 100 mM Hcy (P<0.01). In addition, the relative mRNA expression level of FABP4 in the 100+VB12+FA group decreased significantly (87.69%) compared with the 100 µM Hcy group (P<0.01). The effects of Hcy on the protein levels of FABP4 (Fig. 2B) were measured using western blot analysis, which showed similar results to the mRNA levels. The protein expression levels of FABP4 in the 100 and 200 µM Hcy groups were 3.27 and 2.64 times higher, compared with that in the control group (P<0.05), respectively. FA and VB12 also affected the protein expression of FABP4 significantly, and the relative protein expression of FABP4 in the 100+VB12+FA group was significantly decreased by 52.52%, compared with that in the 100 µM Hcy group (P<0.05). However, as the concentrations of Hcy increased, no dose-dependent increase in the mRNA or protein levels of FABP4 were observed. These results indicated that Hcy affected the expression of FABP4 at the transcriptional and translational levels.

Hcy-induced FABP4 DNA hypomethylation in THP-1 macrophages. DNA methylation patterns have been considered a useful molecular marker for AS, and Hcy can have a global effect on DNA methylation and activate various target genes (16). To determine the effects of Hcy on FABP4 DNA methylation and investigate DNA methylation in the promoter region of FABP4, the THP-1 monocyte-derived macrophages in the present study were treated with Hcy at various concentrations for 24 h, and the DNA methylation status of the FABP4 promoter region of the THP-1 macrophages was determined via nMSP. As shown in Fig. 3, an increase in the concentration of Hcy between 50 and 100 mM, and a further increase to 500 mM, led to statistically significant differences in the levels of FABP4 promoter region DNA methylation, compared with the control group. The methylation changes in the 100 mM group were the most marked. It was also found that, in the experimental groups (50, 100, 200 and 500 mM Hcy, and 100+VB12+FA), the levels of FABP4 DNA methylation decreased significantly, by 18.84, 43.38, 23.65, 16.50 and 25.28%, respectively. The level of FABP4 DNA methylation in the 100+VB12+FA group increased significantly (31.97%), compared with the 100 mM Hcy group (P<0.01; Fig. 3). These results indicated that Hcy decreased the level of FABP4 promoter region DNA methylation.

Hcy-induced cellular TC accumulation in THP-1 macrophages. The present study also assessed whether Hcy accelerated cholesterol accumulation in the macrophages. The human THP-1 macrophages were treated with increasing concentrations of Hcy, and intracellular TC accumulation was assessed using a Total Cholesterol kit. Hcy significantly increased intracellular TC accumulation, compared with the control group, and the highest level was observed at a concentration of 100 µM Hcy (P<0.01). However, the Hcy-induced increase in intracellular TC accumulation did not occur in a dose-dependent manner (Fig. 4).

Identification of the inserted constructed recombinant pcDNA3.1-EGFP/FABP4 plasmid. The recombinant vector
encoding FABP4 was constructed, and it was confirmed that the human FABP4 fragment had been successfully inserted into the pcDNA3.1-EGFP fluorescent eukaryotic expression vector. The FABP4 PCR products were 412 bp on the 1.5% agarose gel (Fig. 5A). The 412 bp fragment encoding human FABP4 was inserted into the pcDNA3.1 -EGFP plasmid between the EcoRI and HindIII sites. The successfully constructed recombinant plasmid, pcDNA3.1-EGFP/FABP4, was cut into two fragments using EcoRI and HindIII (Fig. 5B). In addition, the results of the DNA sequencing were identical to the FABP4 sequence reported on GenBank confirming the results.

Transient FABP4 recombinant transfection induces overexpression of the mRNA and protein levels of FABP4 in THP-1 macrophages. The recombinant expression plasmid containing the FABP4 gene was successfully transfected into the THP-1 macrophages, and the effective mRNA and protein expression of FABP4 were also confirmed using RT-qPCR and western blot analyses, respectively. The data demonstrated that the expression of FABP4 was significantly upregulated in the recombinant plasmid group, with the mean mRNA expression of FABP4 in the recombinant plasmid group being 3.90 and 3.85 times higher than those in the normal control group and empty plasmid control group, respectively (P<0.01; Fig. 6A). The protein levels of FABP4 were measured using western blot analysis, which showed similar results to those observed for mRNA expression (Fig. 6B). The protein expression of FABP4 in the recombinant plasmid group was higher, compared with the normal control group and empty plasmid control group, (69.79 and 63.36%, respectively; P<0.01). Therefore, the pcDNA3.1-EGFP/FABP4 recombinant fluorescent eukaryotic expression vector was successfully constructed and effectively expressed in the THP-1 macrophages, which provided an experimental basis for further experiments.

Effect of the FABP4-specific recombinant on intracellular TC contents. The present study subsequently investigated whether FABP4 accelerated cholesterol accumulation in the macrophages. The human THP-1 macrophages were transfected with the pcDNA3.1-EGFP plasmid and pcDNA3.1-EGFP/FABP4 recombinant plasmid, respectively, and TC levels were examined. As shown in Fig. 7, intracellular TC accumulation in the recombinant plasmid group was significantly higher, compared with that in the normal control group and empty plasmid control group (P<0.01). These results indicated that the overexpression of FABP4 significantly increased intracellular TC accumulation. These observations were consistent with a
previous report which suggested a critical role for FABP4 in macrophage lipid accumulation (17).

Discussion

Previous reports have demonstrated that elevated levels of Hcy have been associated with a number of disease states, including AS (18,19). Despite the important contribution of Hcy to accelerated AS, the specific molecular mechanisms in response to Hcy within macrophages, which is important in atherogenesis, remain to be fully elucidated.

FABP4 is a 15 kDa cytoplasmic protein binding hydrophobic ligands. Deficiency of FABP4 enhances CD36-mediated lipoprotein entry and, at the same time, activates ABCA1-dependent lipid efflux to a greater extent, thereby lowering intracellular lipid contents (20). In the present study, it was found that the exposure of THP-1 macrophages to Hcy was associated with a significant elevation in the expression of FABP4, reaching its maximum level when 100 µM Hcy was used. It was also found that the enhanced expression of FABP4 was paralleled with increases in intracellular TC levels. The present study demonstrated that the exposure of macrophages to Hcy appeared to increase intracellular FABP4 levels and lipid contents, similar to observations in the transformation of macrophages into foam cells in the presence of ox-LDL stimulation (21). In addition, Hcy can be recycled into methionine or converted into cysteine with the assistance of FA and VB12 (22), and mild doses of Hcy inhibitors, including VB12 and FA, markedly attenuated FABP4-induced macrophage lipid accumulation. These findings indicated that the Hcy inhibitors suppressed exclusively pathological excessive lipid accumulation in the macrophages.

To further understand the role of FABP4, the present study constructed the pcDNA3.1-EGFP/FABP4 recombinant vector and successfully transduced the FABP4 gene into macrophages derived from THP-1 monocytes. The increased expression of FABP4 from the transfected macrophages was confirmed using RT-qPCR and western blot analyses. In addition, cellular TC accumulation was examined, and it was found that the intracellular TC content in the FABP4 recombinant plasmid group was also significantly increased. Thus, the increase in cellular TC accumulation was accelerated by the enhanced expression of FABP4, either directly or indirectly. These results suggested that FABP4 may be a potential therapeutic target for the treatment of AS.

Previous population studies have revealed that genetic variations at the FABP4 locus in humans lead to lowered serum triglyceride levels, and a markedly reduced risk of
coronary heart disease (23,24). However, the exact molecular mechanisms remain to be elucidated. In our previous study, Hcy was found to be involved in the disturbance of the expression of the LDL-receptor promoter region and global genome DNA hypomethylation through the interference of DNA methylation (25-27). Increasing evidence also suggests that aberrant DNA methylation around promoter regions can lead to the development of AS (28). Therefore, the present study investigated the epigenetic mechanisms by which the elevated levels of FAPB4 and lipids were affected by Hcy. The resulting data showed that the FABP4 promoter was hypomethylated following exposure to Hcy. These results indicated a causal association between the expression of FABP4 and the changes in DNA methylation, and the epigenetic mechanism of Hcy-induced AS was ascertained.

In conclusion, the results of the present study demonstrated that Hcy not only promoted the secretion of FABP4, but also induced FABP4 gene DNA hypomethylation in cultured...
human THP-1 monocyte-derived macrophages. The invasion of monocytes into the arterial wall and their subsequent differentiation into cholesterol-laden macrophages is a central feature of AS (29). Therefore, the present study examined the effects of Hcy on FABP4, and demonstrated a causal molecular link between Hcy and FABP4 in macrophage lipid accumulation. The most notable finding of the present study was that, Hcy was a stimulator of FABP4 DNA methylation in cultured macrophages, a critical cell in atherogenesis, which induced FABP4 DNA hypomethylation. The results of the present study also confirmed the mechanism that the overexpression of FABP4 accelerates TC accumulation, and thereby promotes the atherosclerotic process. Further investigations, including the analysis of lipid trafficking in macrophages from FABP4-deficient mice and inhibition of lipoprotein transporters by specific small interfering RNA or antibodies, are required to further elucidate the mechanisms.

Acknowledgements

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References