DNA Methylation Polymorphisms Precede Any Histological Sign of Atherosclerosis in Mice Lacking Apolipoprotein E*

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The present work investigates the occurrence and significance of aberrant DNA methylation patterns during early stages of atherosclerosis. To this end, we asked whether the genetically atherosclerosis-prone APOE-null mice show any changes in DNA methylation patterns before the appearance of histologically detectable vascular lesion. We exploited a combination of various techniques: DNA fingerprinting, in vitro methyl-accepting assay, 5-methylcytosine quantitation, histone post-translational modification analysis, Southern blotting, and PCR. Our results show that alterations in DNA methylation profiles, including both hyper- and hypomethylation, were present in aortas and PBMC of 4-week-old mutant mice with no detectable atherosclerotic lesion. Sequencing and expression analysis of 60 leukocytic polymorphisms revealed that epigenetic changes involve transcribed genic sequences, as well as repeated interspersed elements. Furthermore, we showed for the first time that atherogenic lipoproteins promote global DNA hypermethylation in a human monocyte cell line. Taken together, our results unequivocally show that alterations in DNA methylation profiles are early markers of atherosclerosis in a mouse model and may play a causative role in atherogenesis.

Atherosclerosis and its complications are a major cause of death and disability in the developed world. The disease is characterized by infiltration of lipid particles in the arterial wall, accompanied by the recruitment of inflammatory and immune cells, migration and proliferation of smooth muscle cells (SMC),1 and synthesis of extracellular matrix. These processes eventually result in the gradual development of an elevated lipid-rich, fibrocellular lesion (1).

In mammals, DNA methyltransferases use S-adenosyl methionine (SAM) as a methyl group donor to methylate the carbon in position 5 of cytosine residues in a CpG dinucleotide (CG) context (2). DNA methylation regulates fundamental biological phenomena such as gene expression, genome stability, mutation rate, genomic imprinting, and X chromosome inactivation (3–6). Both global and gene-specific alterations in DNA methylation are associated with abnormal phenotypes in disease (7, 8). For example, cancer cells show global genomic hypomethylation and dense hypermethylation of CpG islands, which are normally unmethylated (9). The identification of cancer type- and stage-specific changes in DNA methylation has justified hopes for novel diagnostic and therapeutic avenues (10).

Two general observations suggest that alterations in DNA methylation patterns are involved in atherogenesis (11–13). First, global hypomethylation and dense hypermethylation of certain CpG islands are associated with aging, a major risk factor for atherosclerosis (14). Second, hyperhomocysteinemia and the subsequent decreased production or bioavailability of SAM is associated with an increased risk of cardiovascular disease (15). Accordingly, mice with genetically reduced levels of methylenetetrahydrofolate reductase, a key enzyme in the pathway generating SAM, show hyperhomocysteinemia, DNA hypomethylation, and aortic lipid infiltrations (16). Furthermore, a global DNA hypomethylation has been observed in vascular lesions and leukocytes of atherosclerosis patients and proliferating SMC in animal models (17–19).

One unresolved issue is whether DNA methylation patterns are altered at early stages of atherosclerosis and are associated with susceptibility to the disease. Answers to these questions may have important implications for prevention and therapy of atherosclerosis. We therefore asked whether changes in DNA methylation patterns occur prior to the appearance of any vascular lesions in PBMC and aorta of mice lacking APOE, compared with matched WT animals (20). Moreover, in an attempt to provide a mechanism for the changes in DNA methylation patterns observed in vivo, we asked whether atherogenic lipoprotein profiles could affect DNA methylation and histone post-translational modifications in the human monocyte-macrophage cell line THP-1 (21). The present study is the first analysis of DNA methylation at early stages of atherosclerosis and the first description of genomic DNA sequences undergoing epigenetic changes in a mouse model. The implications of our findings for understanding human atherosclerosis are discussed.

The abbreviations used are: SMC, smooth muscle cells; °C, 5-methylcytosine; CG, CpG dinucleotide; BMP, DNA methylation polymorphism; HPCE, high performance capillary electrophoresis; MSAP, methylation-sensitive amplified polymorphism; SAM, S-adenosyl methionine; PBMC, peripheral blood mononuclear cell; WDMP, wild type DNA methylation polymorphism; MDMP, mutant DNA methylation polymorphism; UK, United Kingdom; EST, expressed sequence tag; WT, wild type; LDL, low density lipoprotein; VLDL, very low density lipoprotein; HDL, high density lipoprotein; HL, high VLDL + LDL mixture; WL, low VLDL + LDL mixture.
**EXPERIMENTAL PROCEDURES**

**Animal Work and Tissue Manipulation**—All procedures used in this study were approved by the local ethical committee (Malmö/Lunds Djurskötska Nämnd, license M911/01). Apoe nullizygous (Apoe<sup>−/−</sup>) of the strain created in the laboratory of N. Maeda (20) were purchased from M&B (Ry, Denmark) and were at the 10th generation of breeding on to the C57BL/6 genetic background. Four-week- and 6-month-old Apoe<sup>−/−</sup>- or C57BL/6 control mice were sacrificed and dissected following overnight fasting.

Dissections were performed according to the anatomy of the mouse (21). The aortic tissue used in this study included the thoracic portion of the middle of the aortic arch and the abdominal portion to the iliac bifurcation. The initial portion of the ascending aorta was used to assess the presence of fatty lesions as described (23). PBMC were isolated by Ficoll-Paque PLUS gradient according to the instructions from the manufacturer (Amersham Biosciences, Little Chalfont, United Kingdom (UK)). Skeletal muscle tissue was dissected from the pelvic limb. Genomic DNA or total RNA were extracted by using the DNeasy or RNeasy system (Qiagen, Valencia, CA), respectively, according to manufacturer instructions. The levels of plasma cholesterol and triglycerides were measured as described (23).

**Methylation-sensitive Amplified Polymorphism (MSAP) Analysis**—DNA methylation profiles were analyzed by the MSAP as reported (24) with the following modifications.

**Restriction Digest of Genomic DNA and Attachment of Adaptors**—All enzymes used in the MSAP protocol were provided by New England Biolabs (Hertfordshire, UK). Five hundred nanograms of genomic DNA were digested for 1 h with 20 units of HpaII and EcoRI using NEB buffer 1 in a 30 µl reaction volume. Subsequently, the restriction digest and ligations were carried out simultaneously for an additional 4 h at 37 °C in a final volume of 40 µl. The restriction-ligation mix contained 10 units of HpaII, 10 units of EcoRI, 10 units of T4 DNA ligase, 5 pmol EcoRI adaptor, 50 pmol HpaII adaptor and 1 mm ATP. The enzymes were inactivated for 15 min at 65 °C, and a second digest was performed for 1 h with 5 units of HpaII and EcoRI, again followed by heat inactivation. The adaptor sequences were as follows: EcoRI adaptor, 5'-CTCTGACTGCGCATGCGCC-3' and 5'-AATTGTTACGAGTCTAC-3; HpaII adaptor, 5'-GATCATGAGTCCTGCT-3'.

**Preamplification**—The preamplification reaction was performed with primers complementary to the core of the adaptor sequences and the target sequence of EcoRI and HpaII restriction enzymes. The sequences of the EcoRI preselective primer (EcoRI-00) and the HpaII preselective primers (HpaII-00) were 5'-GAAGCAGCTGATACATTA-3' and 5'-TCTAGATGTCTGGCTCC-3', respectively. Two microliters of the digestion-ligation mix (diluted 1:3) were added to the pre-amplification mix consisting of 1× PCR Buffer, 0.1 mM dNTP, 50 ng of EcoRI-00 primer, 50 ng of HpaII-00 primer, and 1 unit of Tq polymerase (Bio-Rad) for 1 min at 94 °C followed by 15 cycles as follows: 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min.

**Selective Amplification**—The sequence of the selective primers was identical to the preselective primers but included the addition of a number of nucleotides at the 3' terminus. The selective nucleotides of the EcoRI-00 primer were as follows: EcoRI-01, AGT; EcoRI-02, ACA; EcoRI-03, CAC; and EcoRI-04, ACC. The HpaII selective primers were: HpaII-01, TCCT; HpaII-02, TAGC; HpaII-03, CGAA; HpaII-03A, CAGT; HpaII-04, AATT; and HpaII-04A, AACC. Selective PCR was conducted using 2 µl of pre-amplification mix (diluted 1:10) in a 10-µl reaction volume containing 1× PCR Buffer, 0.1 mM dNTP, 50 ng of EcoRI-00 primer, 50 ng of 5'-labeled HpaII-00 primer, and 1 unit of Tq polymerase. The HpaII primer was end-labeled by incubating 50 ng of primer with 50 µCi of [γ<sup>32</sup>P]dATP, 10 units of polynucleotide kinase and 5 µl of 1× OPA buffer (Amersham Biosciences, Buckinghamshire, UK). The reaction was incubated for 1 h at 37 °C, followed by heat inactivation for 15 min at 65 °C. The PCR cycle employed was a standard amplification fragment length polymorphism touchdown protocol (25).

**Polyacrylamide Gel Electrophoresis**—The PCR samples were mixed 1:1 (v/v) with denaturing buffer (98% formamide, 10 mM EDTA, 0.1% bromphenol blue, and 0.1% xylene cyanol) and separated on 6% polyacrylamide sequencing gel (Bio-Rad) for 3 h at 90 volts. Gels were dried and exposed to x-ray film (BioMax, Eastman Kodak Co.) for 1–4 days at −80 °C. For each experiment, two or three independent MSAP reactions, each corresponding to pooled samples from three or four mice, were performed. If the results were reproducible, one sample was used for further analysis. Quantitative analyses of band patterns were conducted on central portions of gels, where resolution was maximal.

**Sso1 Methyl-accepting Assay**—The assay was performed as previously described (26), with the exception that Nonidet P-40 was replaced with Triton and labeled DNA was blotted on a Nytran SuPerCharge membrane (Schleicher & Schuell, Dassel, Germany).

**Isolation and Sequencing of MSAP Bands**—Bands were excised from acrylamide gels, suspended in 30 µl of 0.5× TE buffer, and incubated for 10 min at 65 °C. One microliter of the solution was used in a standard PCR reaction with the appropriate primer combinations. The resulting fragments were cloned in the pCR-TOPO® vector (Invitrogen, Paisley, UK), and three independent clones were sequenced. Homology searches were conducted using the BLAST program (www.ncbi.nlm.nih.gov).

The Cpg ratio was calculated by dividing the number of observed CG dinucleotides by their expected number, calculated with the formula (number of cytosines/number of guanines) × total number of nucleotides.

**Quantification of Total 5-Methylcytosine by High Performance Capillary Electrophoresis (HPCE)**—Quantification of the degree of methylation was carried out as previously described (30, 31). Briefly, genomic DNA (3–5 µg) was obtained by standard procedures and DNA hydrolyzed and carried out with 1.25 µl (200 units) of nuclease P1 for 16 h at 37 °C. Subsequently, alkali-phosphatase activity was inactivated by the addition of alkali-phosphatase and incubated for 1 h. HPCE was performed with a system (PAC™, MDQ, Beckman-Coulter). Fractionation of the relative methylation of each DNA sample was determined as the percentage of 5-methylcytosine (% C) of total cytosines: % C peak area/C (C peak area + G peak area) × 100. A minimum of five independent HPCE runs was performed in duplicate, and three analytical measurements were made per replicate.

**Quantification of Whole Histone H4 Acetylation and Methylation of Lysine 20 by HPCE**—Quantification of acetylation and methylation at lysine 20 of histone H4 was done by a modification of the method.
previously described (32). Individual histone fractions were obtained from cell nuclei (33), and further purified by reversed-phase HPLC (34). The non-, mono-, di-, tri-, and tetra-acetylated forms of histones H3 and H4 and trimethylated H4 at lysine 20 were resolved by HPCE, using an uncoated fused-silica capillary (Beckman-Coulter) (60.2 cm × 75 mm, effective length 50 cm) in a CE system (P/ACE MDQ, Beckman-Coulter). The running buffer was 100 mM phosphate buffer, pH 2.0, containing 0.02% (w/v) HP cellulose. Running conditions were 25°C and operating voltages of 12 kV. On-column absorbance was monitored at 214 nm. Before each run, the capillary system was conditioned by washing with 0.1 M NaOH for 3 min, and with 0.5 M H2SO4 for 2 min, and equilibrated with running buffer for 3 min. Buffers and washing solutions were prepared with Milli-Q water and filtered throughout, and equilibrated with running buffer for 3 min. Buffers and washing solutions were prepared with Milli-Q water and filtered throughout.

RESULTS

Changes in PBMC and Aortic DNA Methylation Profiles Precede Fibrocellular Vascular Lesions in \( \text{Apoe}^{-/-} \) Mice—To study DNA methylation patterns during the progression of atherosclerosis, 4-week-old and 6-month-old Apoe-null (\( \text{Apoe}^{-/-} \)) were used. Mice were fed normal rodent diet, to exclude any confounding contribution of dietary factors potentially affecting DNA methylation (35). At the age of 4 weeks, mutant mice were hypercholesterolemic but lacked any detectable fatty streak or fibrocellular lesions at the ascending aorta or at the aortic arch (Fig. 1, A and B). By contrast, lesions consisting of a fibrocellular intima and a lipid-rich core were detectable in the same portion of the aortic vessel in 6-month-old \( \text{Apoe}^{-/-} \) mice as previously reported (Fig. 1, C and D) (20). Plasma total cholesterol was markedly elevated in \( \text{Apoe}^{-/-} \) mice of both age groups, relative to WT controls (Fig. 1, compare A and C with B and D) (20).

To screen PBMC for DNA methylation polymorphisms (DMPs) between \( \text{Apoe}^{-/-} \) mice and matched controls, we exploited the MSAP fingerprinting technique. This technique is a modification of amplified fragment length polymorphism, a procedure that is based on random amplification of restriction fragments typically generated by digestion of genomic DNA with the EcoRI and Msei restriction enzymes (25). In MSAP, Msei is replaced by the methylation-sensitive enzyme HpaII, blocked by methylation at either cytosine residue in the recognition site 5′-CCGG-3′ (24). Following digestion of genomic DNA, adaptors are attached to restriction sites that have been successfully digested. It follows that the products of the MSAP ligation reaction consist of DNA fragments that are flanked by hypomethylated HpaII and EcoRI sites. Thereafter, two consecutive PCR reactions, a preamplification and a selective amplification, are performed to enrich a subpopulation of the restriction fragments. The primers employed are complementary to the core sequence of adaptors and recognition sites of the restriction enzymes, and the number of nucleotides added to their 3′ terminus determines their selectivity. Typically the number of selective nucleotides is increased in the selective amplification, where one of the two primers is radioactively labeled. This enables the visualization of a subset of restriction fragments by autoradiography following acrylamide gel electrophoresis.

PBMC DNA was screened by MSAP, using 23 different combinations of selective primers, corresponding to a total of ~1,600 bands/group. Parallel MSAP analyses were conducted with Mspl, an isoschizomer of HpaII that is only sensitive to methylation at the external cytosine (methylation at CNG trinucleotide) on one or both strands (methylation on one strand only is referred to as hemimethylation). It follows that an HpaII polymorphism between WT and \( \text{Apoe}^{-/-} \) samples can be attributed to differential CG methylation, if the corresponding Mspl profile is monomorphic, i.e., either present or absent in both genotypes (Fig. 2A). In the latter case, the HpaII site is hemimethylated at the external cytosine in both genotypes (lower bands in Fig. 2A) (36). Alternatively, absence of Mspl fragments may result from inefficient amplification of the more complex Mspl amplicons, relative to HpaII products. Nonetheless, the stability of Mspl profiles observed throughout our study argues against this hypothesis. A polymorphism detected with both HpaII and Mspl digestion is indicative of CG methylation or mutation at the targeted Apoe alleles or elsewhere (Fig. 2B).

The analysis revealed that polymorphisms were present at both 4 weeks and 6 months of age (Fig. 3). Although DMPs may contain internal hypermethylated HpaII or EcoRI sites, previous analyses revealed that the vast majority of MSAP products represent HpaII-EcoRI fragments that lack any internal HpaII or EcoRI site (37). Accordingly, in the present study, only 5% of the sequenced MSAP fragments (see below) contained an internal HpaII site and none contained any EcoRI site. This implies that hypermethylation of internal fragment sites affects only marginally, if at all, the assessment of genome methylation status by MSAP band scoring. Two hundred and eight of the 206 polymorphisms observed at 4 weeks and 6 months (counting polymorphisms that are common to both ages only once) represented changes in CG methylation, e.g., genuine DMPs (Fig. 3, A and B), whereas only one band represented a difference in CNG methylation or a mutation (Fig. 3C).

The total number of DMPs in 4-week-old PBMC was 48, or 3.2% of total bands. A similar analysis conducted on 6-month-old mice revealed that the total number of DMPs was increased by 3.8-fold to 185, or 11% of total bands, compared with the younger age group (\( p < 0.002 \)). DMPs that were present in both age groups represented a substantial part of all DMPs at 4 weeks (27 of 48, or 56%), but were a minority at 6 months (15%).

An identical MSAP analysis was conducted on aortic DNA, using 14 selective primer combinations, producing a total of ~1,000 bands. Aortic DMPs showed a markedly different dis-
methylation at the flanking HpaII site, respectively, in Apoe<sup>−/−</sup> mice compared with WT. Previous comparisons of parallel results obtained with MSAP and direct quantitation of <sup>31</sup>C by HPCE, established that the total number of bands produced by MSAP accurately reflects the genome-wide methylation status of a particular DNA sample (37). Therefore, the relative proportion of MDMPs and WDMPs allows the estimation of the level of hypo- or hypermethylation of Apoe<sup>−/−</sup> DNA.

Both MDMPs and WDMPs were observed in all samples analyzed, albeit at a different relative extent in different tissues and age groups, indicating that hypo- and hypermethylation occurred simultaneously in Apoe<sup>−/−</sup> DNA (Table I). In 4-week-old mice, the relative proportion of MDMPs and WDMPs was similar in PBMC, whereas a significant excess of MDMPs was observed in the aorta (Table I). By contrast, MDMPs were significantly more frequent than WDMPs in both PBMC and aorta of 6-month-old mice (Table I). The net extent of Apoe<sup>−/−</sup> DNA hypomethylation, was measured by dividing the difference between the number of MDMPs and WDMPs by the total number of bands. DNA hypomethylation did not exceed −4% and was higher in the aorta compared with PBMC (Table I).

To corroborate the result of the MSAP analysis, we assessed the relative methylation status of Apoe<sup>−/−</sup> and control DNA, by the SssI methyl-accepting assay. SssI methyltransferase is a bacterial enzyme that transfers methyl groups to cytosine residues in a CG dinucleotide context, using SAM as a methyl group donor. If labeled SAM is used in the reaction, the incorporation of radioactivity in DNA is proportional to the extent of initial hypomethylation. In accordance with the data obtained by MSAP, 4-week-old Apoe<sup>−/−</sup> aortic, but not PBMC DNA, showed a significant decline in global methylation, whereas hypomethylation was detected in both samples of older Apoe<sup>−/−</sup> mice (Fig. 4, A and B). By contrast, no significant difference in liver or skeletal muscle DNA methylation was detected between Apoe<sup>−/−</sup> and control mice (Fig. 4, A and B). Furthermore, the SssI methyl-accepting assay allows to estimate the number of demethylated CG sites in Apoe<sup>−/−</sup> mice relative to controls, and therefore yields an estimation of the relative extent of global DNA hypomethylation. Again in accordance with the results of MSAP, DNA hypomethylation was more pronounced in the aorta, compared with PBMC (0.40 × 10<sup>6</sup> (n = 5) and 0.64 × 10<sup>6</sup> (n = 12) demethylated CG sites/haploid genome, respectively) at 6 months, and was lower than either sample in aortas of 4-week-old mice (0.25 × 10<sup>6</sup> (n = 12); compare with values in Table I).

Characterization of Variably Methylated Sequences—To characterize the PBMC DMPs, MSAP fragments were excised from acrylamide gels, reamplified by PCR, cloned, and subjected to sequencing in triplicate. The following DMPs were chosen: all the age-independent DMPs, all 4-week-specific DMPs, and 29 randomly chosen 6-month-specific DMPs. Sixty
bands in the 1–4-kb range after digestion with HpaII, is a landmark of global genomic hypomethylation (39). However, no obvious hypomethylation of LINE-1 sequences was detected in the aortic tissue of 6-month-old Apoe−/− mice (data not shown).

Southern Blotting and HpaII-sensitive PCR Analysis of DMPs—DMP profiles and their tissue specificity were verified by Southern blotting and HpaII-sensitive PCR of seven randomly chosen PBMC DMPs. Representative results of four DMPs are shown in Fig. 5. The WDMP M27 is a 304-bp MSAP fragment that was hypermethylated in both 4-week-old and 6-month-old Apoe−/− mice. Accordingly, Southern blotting analysis revealed that the M27 probe hybridized to a band of approximately the expected size in WT PBMC DNA digested with HpaII and EcoRI, and in all samples digested with MspI (Fig. 5A, arrow), whereas it hybridized to higher size fragments in Apoe−/− DNA from both age groups. By contrast, hybridization of M27 to liver DNA-digested HpaII and EcoRI did not reveal any polymorphism (Fig. 5A). In HpaII-sensitive PCR, genomic DNA digested with HpaII was amplified with a primer internal to the DMP fragment, and an external primer, deduced by data base match sequences. Because primers were placed at opposite sides of the DMP-flanking HpaII site, a product could be obtained only if the corresponding HpaII site was methylated. Apoe−/− tail DNA was used as control to assess the tissue specificity of DNA methylation patterns. The analysis showed that the amplification of MDMPs M4 and M54 was reduced in 4-week-old Apoe−/− PBMC, relative to matched WT PBMC or tail DNA, in accordance with their hypomethylated status in Apoe−/− mice (data not shown).

Effects of Lipoproteins on DNA Methylation and Histone H4 Modifications in THP-1 Cells—The occurrence of both local hyper- and hypomethylation of Apoe−/− DNA at the initial stages of atherosclerosis suggests that at least a subset of the observed changes in DNA methylation profiles are active cellular responses to proatherogenic factors. The latter may in-

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**TABLE I**

Hypo- and hypermethylation in Apoe−/− mice

| Type of DMP | PBMC 4 weeks | PBMC 6 months | Aorta 4 weeks | Aorta 6 months | p values refer to differences in DMP counts (χ² test). NS, not significant (p > 0.05). NC, not calculated. |
|-------------|--------------|---------------|---------------|---------------|----------------------------------------------------------|
| MDMPs       | 26           | 111           | 45            | 62            | NS < 0.04                                                |
| WDMPs       | 22           | 74            | 31            | 23            | < 0.02                                                  |
| p           |              |               |               |               | < 0.003                                                  |
| Extent of net DNA hypomethylation (%) | NC | 2.5 | 1.4 | 4.1 | |

**TABLE II**

PBMC DMP sequences with significant matches in data bases

| Clone | Size (bp) | Accession no. | Type of sequence |
|-------|-----------|---------------|------------------|
| M1    | 152       | BM875787      | EST              |
| M4    | 253       | AC132863      | SINE             |
| M7    | 167       | AC115816      | Genomic          |
| M12   | 131       | AC024607      | Genomic          |
| M26   | 301       | AC122350      | LTR/ERVK         |
| M27   | 303       | AC102506      | Genomic          |
| M30   | 216       | BK322548      | Genomic          |
| M42   | 157       | AL607763      | Genomic          |
| M45   | 107       | AC135019      | Genomic          |
| M46   | 285       | AC122350      | Genomic          |
| M49   | 141       | BY249272      | EST              |
| M50   | 61        | AC140236      | Genomic          |
| M52   | 223       | AC130322      | Genomic          |
| M54   | 383       | AC120437      | Genomic          |
| M55   | 226       | AC101290      | Genomic          |
| M58   | 236       | LINE           |                  |
| M62   | 295       | LTR            |                  |
| sz44  | 317       | AC103664      | Genomic          |
| sz47  | 181       | LINE           |                  |
| sz23  | 89        | AC129780      | Genomic          |
| sz7   | 543       | SINE           |                  |
| sz9   | 458       | AC101947      | Genomic          |
| szc13 | 340       | AC115970      | Genomic          |
| szc14 | 343       | LINE           |                  |

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Fig. 4. Genome hypomethylation is tissue-specific in Apoe−/− mice. Figure is a SssI methyl-accepting assay showing the extent of incorporation of tritiated methyl groups into DNA from 4-week-old (A) and 6-month-old (B) mice. Each data point represents an individual mouse. The analyzed tissue and sample size are indicated at the bottom of the figure. For each tissue, data points relative to Apoe−/− and WT DNA are on the left and right side, respectively. Significantly different samples are indicated by asterisks.
include deranged levels of lipoproteins, because hyperlipidemia plays a pivotal role in the initiation of atherosclerosis and precedes the appearance of vascular lesions in Apoe<sup>−/−</sup> mice. To verify this hypothesis, we stimulated the human monocytic THP-1 cells with a mixture of VLDL, LDL, and HDL, and explored the effects on global genomic DNA methylation, by measuring the relative content of <sup>100.9</sup>H<sub>9262</sub>. To further understand the effects of lipoproteins on chromatin structure, we analyzed two post-translational modifications of the histone H4, namely acetylation and methylation at lysine 20 (Lys-20). H4 acetylation is generally associated with a transcriptionally active state, whereas deacetylation is associated with gene silencing and heterochromatin formation (40). Furthermore, trimethylation of Lys-20 of H4 has been associated with silent chromatin and aging (41, 42). Increasing doses of VLDL and LDL had no statistically significant effect on either histone modification (Kruskal-Wallis analysis of variance), whereas differentiated cells showed a significant decrease in histone H4 acetylation and an increase in H4 tri-/dimethyl-Lys-20 ratio, both landmarks of heterochromatin formation (p < 0.0003 for both, Scheffe test, Fig. 6). Overall, the triple global pattern of hypermethylation of DNA and H4, and loss of histone acetylation observed in the cells treated with atherogenic lipoproteins, suggest that gene silencing and the formation of heterochromatin are early molecular "hits" that precede, and probably contribute to, the histopathological traits associated with atherosclerosis.

Fig. 5. Analysis of DMPs by Southern blotting and HpaII-sensitive PCR. A, Southern blot analysis conducted with a probe corresponding to PBMC DMP M27. The arrow indicates the position of genomic hybridizing fragment corresponding to the MSAP DMP. Size of DNA markers are shown on the right (kb). B, HpaII-sensitive PCR analysis of the indicated PBMC DMPs at 4 weeks. Expected sizes of PCR products were 282, 327 and 399 bp, respectively. Size of DNA markers is shown on the right of gel (bp). Abbreviations are as in the legend of Fig. 3.

Fig. 6. Effects of lipoproteins on DNA methylation and histone H4 post-translational modifications in THP-1 cells. Panels show box and Whisker plots of the effects of the WL and HL lipoprotein mixtures on <sup>100.9</sup>H<sub>9262</sub> content (A), levels of acetylated histone H4 (B), and ratio between tri-methylated and di-methylated histone H4 lysine 20 (C) in undifferentiated and differentiated THP-1 cells. Control, incubation without lipoproteins. HL and WL, high and low VLDL+LDL lipid mixtures, respectively, as described under "Results." The average of triplicate measurements, with S.D. and S.E., are shown for each sample. Asterisks represent the significance levels compared with matched controls (Scheffe test).
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DISCUSSION

The present work expands the current knowledge on the role of DNA methylation in atherosclerosis, by showing that DMPs are present prior to the appearance of vascular lesions in Apoe−/− mice. Noticeably, DMPs were significantly more frequent in organs and cell types involved in the initiation and progression of atherosclerosis, e.g. the aorta, circulating inflammatory cells, and/or immune cells (43, 44), in comparison with the control tissues examined (liver, pericardial fat, skeletal muscle). Furthermore, few DMPs were common to PBMC and the aorta, mirroring the functional differences between the two tissues. Taken together, the observed mosaicism and tissue specificity of DMPs suggest that these epigenetic marks are specifically associated with predisposition to and progression of atherosclerosis, rather than originating from genetic or stochastic epigenetic variability. An additional factor known to affect DNA methylation patterns is transgene integration, implying that a similar effect may be exerted by the insertion of exogenous sequences into targeted Apoe alleles (45). Nevertheless, the mosaic nature of the observed DMP strongly argues against such a mechanism.

In agreement with previous studies, we observe a modest, but significant global hypomethylation of genomic DNA in aortas and PBMC of Apoe−/− mice afflicted by fibrocellular lesions (18, 19). The extent of aortic DNA hypomethylation observed in our study was lower in comparison with the corresponding value obtained by Hiltunen and colleagues (18) in Apoe−/− mice, humans, and rabbits. These discrepancies have several possible explanations. First, Hiltunen and colleagues assessed the effects of dietary lipids, regardless of the consequences of atherosclerosis per se in Apoe−/− mice (e.g. in comparison with matched WT mice). Similarly, the work conducted by the same group in rabbits analyzed the epigenetic effects of dietary lipids and cell proliferation following arterial denudation, rather than genuine atherosclerosis. Furthermore, the measurement of DNA methylation in human atherosclerotic lesions is potentially affected by inter- and even intra-individual epigenetic variability, particularly in samples that are forcibly of relatively limited size (18, 38). In accordance with the modest net DNA hypomethylation estimated by MSAP, methylation of LINE-1 sequences was not detectably changed in Apoe−/− aortic DNA compared with controls. Taken together, these observations suggest that the mechanism of genome hypomethylation differs in atherosclerosis, cancer, and aging, and that similarities between the epigenetics of these pathophysiological situations must be drawn with caution. Our results suggest that atherosclerosis is associated with a significant rearrangement of DNA methylation patterns, including both hyper- and hypomethylation, in the PBMC and the aorta. The samples analyzed in this study represent heterogeneous cell populations; therefore, more extreme changes in DNA methylation, perhaps including hypomethylation to levels observed in cancer and aging, cannot be excluded to occur in selected cell types. The results of sequencing and expression analysis suggest that at least some of the observed DMPs represent germline sequences.

Correlative studies suggest that DNA hypomethylation may be a passive phenomenon because of the depletion of cellular SAM in hyperhomocysteinemia (19, 46, 47). However, hyperhomocysteinemia is not a trait of Apoe−/− mice, implying that the process is not merely a passive consequence of the depletion of cellular SAM (48). Rather, our results suggest that DMPs are the compound result of active and passive mechanisms. The finding that lipoproteins induce DNA hypermethylation in cultured cells suggests that changes in DNA methylation are among the earliest cellular changes in atherosclerosis. Furthermore, these observations demonstrate for the first time a direct link between a proatherogenic factor (the hyperlipidemic lipoprotein profile) and changes in DNA methylation. Our results are consistent with the observation that the atheroprotective HDL causes chromatin activation in cell culture (49). As the disease progresses, and the involvement of SMC becomes more significant (43), hyperproliferation of the latter cell type in the fibromuscular lesion may then tip the balance of global genome methylation status toward hypomethylation, as previously shown (18). The mechanisms by which lipoproteins can affect DNA methylation patterns are currently not understood. APOA-I, a constituent of HDL, is physically associated with active chromatin and binds to a CG-rich oligonucleotide in vitro, suggesting a role for lipoprotein constituents in chromatin binding and remodeling (50, 51). Further studies are necessary to elucidate the functional consequences of these interactions in atherogenesis.

Incidentally, we show that deacetylation and increased trimethylation of histone H4 lysine 20 occur in differentiated, unstimulated THP-1 cells in the absence of a significant DNA hypermethylation. Further investigations are needed to interpret this result in the light of the current models of heterochromatin formation (52, 53).

In conclusion, we report that specific changes in DNA methylation occur during the early phases of atherosclerosis in a mouse model. Furthermore, by showing that DMPs are present in accessible cell types such as PBMC at early stages of atherosclerosis, our work points to epigenetic marks as potential novel tools for prevention and therapy.

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