DNA Methylation and Atherosclerosis

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ABSTRACT DNA methylation is a major epigenetic modification of the genome that has the potential to silence gene expression. Recently, the role of epigenetic alteration as a distinct and crucial mechanism to regulate genes governing cell proliferation in atherosclerosis has emerged. Aberrant methylation is related to aging, and, because it affects a large number of CpG islands, age-related methylation may be an important contributor to increased atherosclerosis among older individuals by upregulating atherosclerosis-susceptible genes and downregulating atherosclerosis-protective genes. Further dissection of epigenetic alterations in atherosclerosis and aging will lead to the identification of novel epigenetic modifiers and improved diagnosis and treatment for atherosclerosis-related diseases. J. Nutr. 132: 2406S–2409S, 2002.

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Atherosclerotic complications remain the leading causes of mortality and morbidity in Western society. In the United States, cardiovascular disease is responsible for one in every 2.4 (41.4%) deaths and is the leading cause of mortality (1). Enormous efforts have been undertaken to study the risk factors, pathogenesis and signaling pathways involved in atherosclerosis. These conventional approaches have led to the identification of many risk factors that contribute to the development of atherosclerosis, including cigarette smoking, hyperglycemia, hypertension, insulin resistance, excess circulating lipids and inadequate exercise (1). The currently available preventive measures for atherosclerosis-related diseases are consequently based on the modification of these risk factors. In the postgenomic era, in which the entire reference genome sequence and some of its frequent variants are available, a new page of atherosclerosis research concerning the study of genetic alterations has been underway. However, in contrast to cancer, the pathogenesis of which has been linked to base pair change, deletion, insertion, recombination and amplification of oncogenes (genomitis), atherogenesis is believed to involve mainly changes in gene function rather than structural mutations. Such functional changes may result from alterations in gene expression or activity of gene products, resulting from polymorphisms, most commonly single nucleotide polymorphisms. In addition, dynamic alterations in gene expression may result from differential epigenetic changes, particularly DNA methylation.

DNA methylation is the postreplication addition of methyl groups to the 5′ position of cytosine rings within the context of CpG dinucleotides, resulting in alterations in the appearance of the major groove of DNA to which DNA binding proteins bind (2). These epigenetic “markers” on DNA can be copied after DNA synthesis, resulting in heritable changes in chromatin structure, which influence gene activity but do not involve changes in DNA sequences. Approximately 70% of CpG pairs in the mammalian genome are constitutively methylated (3). These methylated regions are typical of the bulk chromatin that is responsible for the silencing of the overwhelming amount of noncoding DNA present in the mammalian genome, including introns, repetitive elements and potentially active transposable elements (4). In contrast to these constitutively methylated regions, there are short stretches of DNA sequences with an unusually high guanine-phosphate-cytosine (GC) content and a high frequency of CpG dinucleotides, ranging from 0.5 to 5 kb and occurring on average every 100 kb, which are unmethylated. These unmethylated sequences are called CpG islands (5). CpG islands are GC rich (60–70%) and have a ratio of CpG:GpC of at least 0.6 (6). Collectively, CpG islands account for 1–2% of the genome and their location is primarily in the 5′ regulatory

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regions of all housekeeping genes as well as up to 40% of tissue-specific genes (6). CpG islands have also been proposed to function as replication origins (7). Although they are generally not methylated, CpG islands have been the focus of most investigations into the role of DNA methylation in biologic processes. DNA methylation has been shown to be essential for normal development (8), X chromosome inactivation (9), imprinting (10) and the suppression of parasitic essential for normal development (8), X chromosome inactivation (11). Recent evidence indicates that, in addition to genetic alterations, methylation of CpG islands represents an important mechanism controlling gene expression in diseases such as cancer and atherosclerosis. This article reviews the remarkable progress in the epigenomic atherosclerosis research.

**DNA methylation of antiproliferative genes in atherosclerosis**

Embryogenesis and differentiation are characterized by specific patterns of gene expression in tissues and organs and proceed in the absence of any alterations in DNA sequences. Thus, the diversity of phenotypes in differentiated organs is due not to any genetic alterations but to epigenetic changes. Keeping this in mind, we examined if methylation and subsequent suppression of gene expression occurred in atherosclerosis, where a change from a quiescent, contractile smooth muscle cell (SMC) phenotype to a proliferative, migratory and synthetic phenotype is a key process. The methylation status of estrogen receptor-α (ERα), a gene shown to be methylated in virtually all colonic tumors, was investigated in multiple tissue specimens collected from patients undergoing coronary artery by-pass surgery and in atherosclerotic plaques collected from patients undergoing directional coronary atherectomy or carotid endarterectomy. DNA isolated from these tissues was treated with methylation-sensitive enzyme NodI and subjected to Southern blot analysis, which revealed increased ERα gene methylation in all specimens showing atherosclerosis. Furthermore, in atheromas, ERα methylation was significantly increased when compared with normal proximal aortas (12). These findings, for the first time, linked DNA methylation of CpG islands to cardiovascular disease.

To determine whether the cells responsible for ERα methylation were SMCs in atherosclerosis, we established a SMC dedifferentiation model, a model in which SMCs become proliferative and noncontractile as occurring in atherosclerotic lesions in vivo. In this model, SMCs directly isolated from normal human aortas (representing contractile phenotype) were compared with the same cells explanted from the aorta and cultured in vitro (resembling the in vivo dedifferentiated phenotype) (13). Restriction landmark genome scanning (RLGS), a highly resolving two-dimensional electrophoresis-based technique capable of identifying genome-wide methylation changes in CpG islands, was used to study the methylation profiles of these cells. Consistent with Southern blot data obtained from our in vivo study, ERα was indeed among the spots present in contractile but not proliferative SMCs, indicating that methylation of ERα had occurred during the phenotypic switch (13). ERα methylation was further confirmed by combined bisulfite restriction analysis of these SMCs. In contrast, human aortic endothelial cells did not show differential methylation of ERα in culture. Moreover, changes in the methylation status of CpG islands were found in 11 additional genes in proliferating SMCs as compared with in situ SMCs (13). These changes represented ~0.5% of the CpG islands screened, indicating that CpG island methylation is not an infrequent event during the dedifferentiation process of vascular SMCs.

ERα, upon activation by estrogen, regulates a variety of cellular activities, including the inhibition of cell proliferation observed in cancer cells sensitive to estrogen. Such an antiproliferative effect could also slow the proliferation of SMCs that occurs within the blood vessel wall in response to injury (14,15). Moreover, activated ERα increases the expression/activity of nitric oxide (NO) synthase, resulting in enhanced NO production that, in turn, could inhibit SMC proliferation and platelet activation. These effects of ERα may account for the cardiovascular protection afforded by estrogen, contributing to the low incidence of atherosclerotic disease in premenopausal women and accelerated atherosclerosis in postmenopausal women. In addition, our findings may help explain the failure of hormone replacement therapy (HRT) as observed in the heart and estrogen/progestin replacement study, in which women with preexisting coronary artery disease were found to gain no clinical benefits following 4 y of HRT, in spite of improved lipid profile for the HRT recipients (16). Methylation-induced silencing of the ERα gene with atherosclerosis in these women may have accounted for their “resistance” to the protective effects of estrogen.

p53 is another gene whose methylation status was investigated in relation to atherosclerosis (17). As a tumor suppressor protein, p53 has both antiproliferative and proapoptotic actions. Embryonic fibroblasts from p53 knockout mice display higher proliferative rates compared with cells from wild-type mice. When such p53 knockout mice were crossed with atherosclerosis-susceptible apolipoprotein E (apoE) knockout mice, the double-knockout offspring exhibited a statistically significant 50–100% increase in atherosclerosis lesions at 6, 10, and 15 wk of age as compared with p53-positive apoE knockout mice, and such lesions were hypercellular. Bromodeoxyuridine incorporation (for detecting proliferation) and in situ DNA extension (terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling) assay (for detecting apoptosis) studies revealed that increased cellular proliferation, and not decreased apoptosis, contributed to the aggravated lesion development in the double-knockout mice (18). These results indicate that p53 exerts its antiatherosclerotic effect via inhibiting cell proliferation. Interestingly, Rodriguez-Campos et al. (18) demonstrated that the expression of p53 protein and mRNA was completely downregulated in cultured tunica media from explanted porcine aortas relative to contractile medial SMCs residing in the healthy vessel. This downregulation, however, was not due to mitogen-dependent methylation of the 5’ control region of p53, suggesting that SMC dedifferentiation and proliferation may require mitogen-induced suppression of p53, which is not mediated by methylation.

To date, ERα represents the only gene known to have differential CpG island methylation in its promoter region in atherosclerosis. The major function of estrogen is to regulate SMC proliferation. Because atherosclerosis is a multifactorial complex trait, it is likely that multiple genes govern the phenotypic switch of SMCs in atherogenesis. In addition to proliferation, genes involved in processes such as extracellular matrix accumulation, energy metabolism and intracellular milieu maintenance, processes important for atherogenesis, may be regulated by DNA methylation. Indeed, through a large-scale RLGS screening for candidate genes that may be methylated or demethylated in the promoter region in association with aging and atherosclerosis in a large number of aortas, we have identified additional factors involved in SMC proliferation and multiple genes with various cellular activities (19).
Moreover, Laukkanen et al. (20) detected a marked reduction in the amount of methylated CpG dinucleotides in the coding region of extracellular superoxide dismutase gene in atherosclerotic aortas as compared with normal aortic media in a rabbit model of atherosclerosis. Because methylation/demethylation in the transcribed region may not affect transcription, the significance of this finding remains to be determined.

**DNA methylation: the missing link between aging and atherosclerosis?**

Aging and atherosclerosis are considered major independent risk factors for cardiovascular morbidity and mortality. Epidemiological studies reveal that the clinical sequelae of atherosclerosis-related conditions all increase with age (21-22). Autopsy studies demonstrate that atherosclerosis in the vascular beds supplying blood to the heart, brain, kidneys and lower extremities increases with age (23). Thus, aging and atherosclerosis are closely related and operate simultaneously to affect the cardiovascular system adversely. There is, however, little understanding of the mechanisms underlying the relationship between aging and atherosclerosis. In particular, although it has been suggested that there is a progressive drift of gene expression occurring with aging, of the cause of such a drift remains an enigma.

The identification of CpG methylation as a potential mechanism of epigenetic inheritance has led to speculation that it might be involved in the aging process (24). Progressive losses of 5-methylcytosine levels have been observed in normal fibroblasts during in vitro passaging (25,26). Similarly, genomic hypomethylation of bulk chromatin has been described in aged tissues in vivo (27-29). Recent studies have demonstrated age-related hypermethylation at selected loci, including ERα, c-Myc, c-Fos, insulin-like growth factor-II, MYODI, N33, HIC1, versican, PAX6, and E-cadherin and P15 (30). Importantly, many genes that had previously been thought to be methylated exclusively in cancer have now been found to belong to the group of age-related methylated genes. In fact, several studies indicate that age-related methylation is a common cause of hypermethylation in cancer, accounting for as much as 50-80% of all such occurrences (30). We have found that ERα methylation in heart muscle correlated with age (12). In cultured SMCs, ERα methylation ranged from 19% in one sample obtained from an infant to 99% in a sample obtained from the cadaver of an adult at the same passage number (13). Thus, age-related methylation is not an exclusive property of cancer; rather, it may contribute to other age-related diseases, including atherosclerosis. In addition to gene-specific hypermethylation, age-related global hypomethylation has been observed and is linked to genomic instability and increased mutation rates. Importantly, atherosclerotic aortas were found to have decreased 5'-methylcytosine content in the genome, as compared with normal arteries in a rabbit model of atherosclerosis (20). Thus, it appears that age-related global hypomethylation and gene-specific hypermethylation may have profound impacts on the pathogenesis of atherosclerosis.

**DNA methylation, hyperhomocysteinemia and atherosclerosis**

Homocysteine is a sulfur-containing amino acid, derived from methionine, which can be remethylated back to methionine as part of the methionine cycle. In this cycle, methionine is used for synthesis of S-adenosylmethionine (SAM), which is converted to homocysteine and S-adenosylhomocysteine (SAH), a potent methylation inhibitor. SAM is the methyl donor for >100 different transmethylation reactions, including DNA methylation (31). Mice deficient for methyltetrahydrofolate reductase, which converts 5,10-methyltetrahydrofolate to 5-methyltetrahydrofolate, a methyl donor for homocysteine remethylation to methionine, had significantly increased total plasma homocysteine levels (1.6-fold for heterozygotes and 10-fold for homozygotes) compared with wild-type littersmates. They also displayed either significantly decreased SAM levels or significantly increased SAH levels. These mice showed a statistically significant increase in hypomethylated sites in DNA, suggestive of global hypomethylation. Interestingly, abnormal lipid deposition in the proximal portion of the aorta was present in older heterozygotes and homozygotes (32). Moreover, Wang et al. (33) found that clinically relevant concentrations of homocysteine (10-50 μM), but not cysteine, inhibited DNA synthesis and growth in vascular endothelial cells, and such an inhibitory effect might be mediated through hypomethylation of proteins and genes. Hence, global DNA hypomethylation may serve as a mechanistic link between hyperhomocysteinemia and atherosclerosis. Further work is warranted to characterize genome-specific hypermethylation and hypomethylation as opposed to global hypomethylation in the presence of hyperhomocysteinemia and the significance of such hypermethylation in consequent atherosclerosis.

Despite remarkable progress, the study of DNA methylation in atherosclerosis is just beginning. With the advent of RLGS and CpG island array technologies, now it is possible to perform large-scale analysis to examine epigenotype-phenotype relationships, which may unravel an epigenetic mechanism underlying atherogenesis. We have initiated a substantial endeavor to study epigenetic alteration in a large number of aortas collected from cardiac transplant donors of different ages. Such studies may determine whether DNA methylation serves as a link between aging and atherosclerosis by affecting the drift of gene expression associated with aging, which could lead to the identification of novel atherosclerosis-susceptible or -protective genes.

**LITERATURE CITED**