Inactivation of Monocarboxylate Transporter MCT3 by DNA Methylation in Atherosclerosis

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**Background**—Monocarboxylate transporters (MCTs) mediate lactate transport across the plasma membrane of cells. The molecular mechanisms regulating monocarboxylate transport in smooth muscle cells (SMCs) remain poorly characterized. The aim of this study was to investigate the effects of DNA methylation on MCT expression and lactate transport in SMCs in relation to atherosclerosis.

**Methods and Results**—MCT expression was determined by real-time reverse transcription–polymerase chain reaction, Western blotting, and immunohistochemistry in SMCs isolated from human aortas and coronary arteries. Bisulfite sequencing and confocal microscopic analysis were used to study DNA methylation and lactate transport in SMCs, respectively. Downregulation of MCT3 and impaired lactate transport were detected in proliferating/synthetic SMCs, relative to the contractile phenotype. A passage number– and atherosclerotic lesion–dependent methylation pattern of MCT3 was demonstrated in the CpG island located in exon 2. Treatment of SMCs with the demethylating agent 5-aza-2'-deoxycytidine restored MCT3 expression and normalized lactate transport. Furthermore, small interfering RNA–mediated specific MCT3 knockdown substantially stimulated SMC proliferation.

**Conclusions**—These data indicate that DNA methylation may modify monocarboxylate transport by suppressing MCT3 expression, which could be important in regulating SMC function and the development of atherosclerosis. (*Circulation*. 2005;112:1353-1361.)

**Key Words:** atherosclerosis ■ cardiovascular diseases ■ ion channels ■ molecule biology ■ muscle, smooth

Atherosclerosis is a complex disease characterized by the accumulation of lipids, fibrous materials, cell debris, and minerals in the arteries. Much attention has been focused on characterizing the influence of traditional risk factors, such as lipids, homocysteine, glucose, and recently, cytokines and inflammatory markers, on vascular cells and atherosclerotic lesion development. Little effort, however, has been made to elucidate the effects of monocarboxylates, including lactate, pyruvate, and ketone bodies, and their transport in atherogenesis. Indeed glucose, the preferred energy source in vascular smooth muscle cells (SMCs), is metabolized through aerobic glycolysis, resulting in the production of quantities of lactate, which must be transported from the cell to prevent intracellular acidification. Furthermore, lactate is also utilized by muscle cells as fuel and must be transported into these cells. Hence, transport of lactate and probably other monocarboxylates represents an important metabolic process in vascular cells, in particular SMCs, and inadequate monocarboxylate transport may affect the development of atherosclerotic lesions.

Transport of lactate, pyruvate, and ketone bodies across the plasma membrane is mediated via a family of proton-coupled monocarboxylate transporters (MCTs). Nine members of this family have been cloned in humans. To date, only isoforms MCT1 through 4 have been shown to transport lactate. MCT3, originally cloned from a chicken retinal pigment epithelium (RPE) expression library, is an important member of the MCT family. MCT isoform expression and monocarboxylate transport in human SMCs remain to be characterized.

DNA methylation refers to the addition of a methyl group to the 5 position of cytosine in the context of a CpG dinucleotide. Although controversy still exists about the impact of the position and size of the methylated DNA segments on regional transcription, it has been well established that this epigenetic change influences gene expression. Specifically, DNA methylation is associated with transcription silencing, and loss of methylation (demethylation) activates gene expression. Increasing evidence indicates that human diseases, including cancer and atherosclerosis, are either caused or impacted by abnormal methylation.

In the present study, we investigated MCT3 methylation and expression in cultured SMCs, coronary arteries, and aortas with varying atherosclerotic burden. We show that methylation of a CpG island located in exon 2 of the MCT3...
gene suppresses its transcription, a phenomenon associated with increased passage number of cultured SMCs and with advanced atherosclerotic lesions. MCT3 methylation was associated with impaired lactate transport in SMCs. Importantly, treatment of SMCs with the demethylating agent 5-aza-2'-deoxycytidine (5-aza-dC) restored MCT3 expression and normalized lactate transport. Furthermore, small interfering RNA (siRNA)–mediated specific MCT3 knockdown substantially stimulated SMC proliferation. These data, for the first time, indicate the effect of DNA methylation of the MCT3 gene in the dysregulation of monocarboxylate transport and SMC proliferation in the context of atherosclerosis.

Methods

Tissue Collection and Processing
Aortas of heart donors were collected at the time of organ harvest and stored in University of Wisconsin solution on ice to minimize postmortem changes. For this study, 10 normal aortas were used for isolating SMCs, and 23 aortas and 12 coronary arteries were used for MCT3 expression and/or methylation study. The studies were approved by the institutional review board of the Duke University Medical Center. Detailed tissue processing protocols are presented in the online-only Data Supplement.

Cell Culture
Primary human aortic SMCs were obtained from the tunica media of 10 normal thoracic aortas after surgically dissecting away the adventitia, as described. Detailed protocols are presented in the online-only Data Supplement.

TaqMan Real-Time Reverse Transcription–Polymerase Chain Reaction (RTT-PCR)
RNA was isolated from cultured cells and frozen aortas with the use of an RNeasy mini kit (Qiagen). One microgram of total RNA was used for synthesis of first-strand cDNA with the Superscript preamplification system (Life Technologies). Detailed RTT-PCR protocols are provided in the online-only Data Supplement.

Immunoblotting Analysis
Cell extracts were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis under nonreducing conditions. Proteins on the gel were then transferred to a nitrocellulose membrane. Immunoblotting was performed with rabbit anti-MCT1, 2, 3, and 4 antibodies, followed by incubation with goat anti-rabbit horseradish peroxidase–conjugated secondary antibody (Zymed), and detection by chemiluminescence.

Immunohistochemistry
Cardiac specimens were processed by 10% formalin fixation and paraffin embedding. Immunohistochemistry for MCT3 (Chemicon International) and SM α-actin (Dako) was performed according to protocols provided in the online-only Data Supplement.

Bisulfite Sequencing
Genomic DNA was isolated from the tunica media, cultured SMCs, and aortas with the use of a GIBCO DNazol isolation kit, according to the manufacturer’s instructions. Two micrograms of genomic DNA from each sample was treated with sodium bisulfite in the CpGenome DNA modification kit (Oncor Inc), according to the manufacturer’s instructions. Detailed protocols and primer sequences for PCR amplification after bisulfite treatment are presented in the online-only Data Supplement.
The lack of a compensatory increase in the expression levels of MCT1, 2, and 4 renders MCT3 a probable rate-limiting factor for the putatively compromised monocarboxylate transport in proliferating SMCs residing in atherosclerotic plaques in vivo. Hence, in the following experiments, we focused our investigation on MCT3.

To investigate whether downregulation of MCT3 also occurred in vivo in the context of atherosclerosis, we examined MCT3 mRNA and protein expression by TRT-PCR and Western blotting in 23 aortas showing varying degrees of atherosclerosis collected from cardiac transplant donors. Atherosclerosis burden was evaluated by en face Sudan IV staining and analysis of raised plaques, as described in the PDAY study, and was classified as normal, mild, moderate, or severe. Remarkably, a graded decrease in MCT3 mRNA and protein expression was observed relative to the increased severity of atherosclerosis in these vessels (Figure 2). Considering that the switch of SMCs from the contractile to the proliferating/synthetic phenotype and loss of the tunica media are key events in atherogenesis, these data are highly consistent with our in vitro observations.

To further determine whether the lack of MCT3 expression was correlated with SMC dedifferentiation and medial thinning associated with atherosclerosis, we performed immunohistochemistry with MCT3 antibody in autopsied myocardial tissues from coronary arteries collected from 12 patients who had died of ischemic heart disease. MCT3 expression was detected in both the myocardium and coronary vessels. In coronary arteries, MCT3 expression was restricted to the tunica media, and colocalization of MCT3 and SM α-actin was demonstrated. In the neointima, where 60% of cells were proliferating/synthetic SMCs as indicated by SM α-actin staining, MCT3 was almost undetectable (Figure 3). Remarkably, the majority of MCT3-negative cells colocalized with SM α-actin–positive cells in the neointima. These data indicate that the decreased MCT3 expression in atherosclerosis may be, at least in part, due to loss of the tunica media and SMC dedifferentiation in the disease process.

Methylation Status of MCT-3 in Cultured SMCs and Normal and Atherosclerotic Aortas

To investigate whether DNA methylation might contribute to the decreased expression of MCT3 in cultured SMCs and in atherosclerotic aortas, we first analyzed the structure of the MCT3 gene, in particular, the number and distribution of scattered CpG sites and CpG islands with use of an online database. We found that the CpG islands in the promoter region of the MCT3 gene were methylated in normal aortas but not in atherosclerotic aortas. Furthermore, we examined the methylation status of MCT3 in cultured SMCs and found that the expression of MCT3 was inversely correlated with the methylation level of the CpG islands in the promoter region. These data suggest that DNA methylation might play a role in the regulation of MCT3 expression in atherosclerosis.
Figure 2. MCT3 expression is decreased in aortas with atherosclerosis in a lesion-dependent manner. MCT3 mRNA (A) and protein (B) are decreased in 18 aortas with mild (7), moderate (5), and severe (6) atherosclerosis relative to 5 normal vessels. The expression levels are inversely correlated with the degree of atherosclerosis burden. *P<0.05, **P<0.01.

Figure 3. Decreased MCT3 expression in the vessel wall is associated with SMC dedifferentiation in atherosclerosis. MCT3 expression is exclusively detected in the tunica media of coronary arteries, but not in the neointima (A, C). SM α-actin expression is present in both the tunica media and neointima (B, D), indicating that a majority of cells located in the neointima are dedifferentiated SMCs. Colocalization of MCT3-negative and SM α-actin-positive cells is noted. Magnification, A and B, ×110; C and D, ×330. M indicates media; I, intima; arrows, internal elastic lamina.

program, cpgplot, available at http://www.hgmp.mrc.ac.uk, which defines a CpG island as a 200-bp region with a calculated CG composition >50% and an observed to expected CpG ratio of >0.6. This analysis revealed that although the 2303-bp MCT3 promoter did not contain CpG islands, there were 59 discrete CpG sites scattered in the promoter region. Furthermore, 2 CpG islands, from −10 to +272 (282 bp with 31 CpG sites) and from +1192 to +2053 (862 bp with 105 CpG sites), were located in exon 2 and exon 4, respectively, of the coding region of the MCT3 gene.

To determine the correlation between DNA methylation and expression of MCT3, we chose to examine the methylation status of differentiated SMCs (passage 0) and cultured SMCs at passage 8, extremes of SMCs in terms of MCT3 expression, by genomic bisulfite sequencing, a technique capable of detailed mapping of methylated residues of a specific gene. Although most previous investigations have focused on CpG island methylation in the promoter region, which has been causally associated with transcription silencing, methylation of scattered CpG sites, however, may also affect transcription. This is particularly true if these CpG sites are located in the binding sites for DNA binding proteins, which may prevent efficient protein-DNA interactions and subsequent transcription initiation.

To survey the methylation profiles of the promoter region, we first designed 3 pairs of primers from −1238 to −270 bp, where 27 of the 59 CpG sites are located, to amplify the modified DNAs. The PCR products were ligated, transformed, and further amplified with the pCR 4-TOPO vector and DH5α cells. Five to 8 clones for each cell strain were used for sequencing. Percent methylation of CpG sites for this promoter region in a given cell strain was calculated on the basis of sequencing data derived from all clones. Surprisingly, all 27 CpG sites were uniformly methylated in all clones for each cell strain, and all cell strains exhibited the same methylation pattern regardless of expression status of MCT3 and culture status of the cells used (data not shown). We also examined the methylation status of the MCT3 promoter in human RPE, the only cells that are known to express a high level of MCT3. With use of the same primers, uniform methylation of all 27 CpG sites was similarly detected in the MCT3 promoter in RPE cells. The remaining 32 CpG sites are distributed at widely spaced intervals in the MCT3 promoter, hindering efficient methylation analysis. Nevertheless, the data obtained from these 27 CpG sites surveyed suggest that methylation of discrete CpG sites in the promoter region of MCT3 does not appear to suppress its transcription and cannot be used to explain the observed transcriptional silencing in cultured SMCs relative to differentiated SMCs. The data also rule out the possibility that a cell type–specific MCT3 promoter, distinct from that of RPE, may be operative in SMCs.

Recent evidence indicates that DNA methylation of the coding region is more powerful in suppressing gene expression than the promoter region.12,13 Although controversial, this represents a significant progress in our understanding of the effects of DNA methylation. To investigate DNA methylation of CpG islands in the coding region of MCT3, 2 pairs of primers covering the CpG island in exon 2 and 3 primer pairs specifying the CpG island in exon 4 were used, and genomic bisulfite sequencing of the amplified products was performed. The percentage of CpGs located in each CpG island in a given cell strain was calculated on the basis of sequencing data derived from 5 to 8 PCR product clones. In SMCs at passage 8, complete methylation of CpG dinucleotides in 81±9% of the clones for the CpG island located in exon 2 was demonstrated in 7 of the 10 cell strains where
MCT3 expression was absent. The remaining 3 SMC strains showed partial methylation (65±6%) of CpG dinucleotides in 76±4% of the clones analyzed (Figure 4). In contrast, in 6 of the 10 normal tunica media (SMCs at passage 0), where MCT3 expression was abundant, no DNA methylation was detected in 73±12% of the clones for each sample. The remaining 4 had partial DNA methylation (25±9%) of CpG dinucleotides in 30±7% of the clones for each cell strain (Figure 4). Bisulfite sequencing analysis of the CpG island located in exon 2 of the MCT3 gene was performed on 5 to 8 clones for each of the 10 SMC strains. The percentages of methylated CpG dinucleotides, affected clones, and involved SMC strains were calculated. The data show markedly increased DNA methylation status in SMCs at passage 8 relative to passage 0. **P<0.01.

Reactivation of MCT3 Expression by 5-aza-dC

If transcriptional silencing of MCT3 were indeed attributed to DNA methylation, in particular, the CpG island located in exon 2, then demethylating the gene with 5-aza-dC would restore MCT3 expression in cultured SMCs. To test this hypothesis, we selected 4 SMC strains at passage 8 that showed complete exon 2 CpG island methylation in >90% of the clones and a lack of MCT3 expression by both TRT-PCR and Western blotting. Cells were treated with different concentrations (1 μmol/L and 5 μmol/L) of 5-aza-dC for 4 days, and the medium containing 5-aza-dC was replaced daily. As shown in Figure 6A and 6B, in all SMC strains, MCT3 mRNA and protein expression were induced by 5-aza-dC in a dose-dependent manner, whereas no induction of MCT1, 2, and 4 mRNA expression was detected (data not shown). To confirm that reactivation of MCT3 expression by 5-aza-dC was mediated by DNA demethylation in exon 2, genomic DNA was isolated from 3 of 4 SMC strains before and after 5 μmol/L 5-aza-dC exposure and subjected to bisulfite sequencing. As expected, all CpG dinucleotides in the exon 2 CpG island in these SMCs became demethylated (Figure 6C). Furthermore, 5-aza-dC treatment of the tunica media and RPE did not further increase the level of MCT3 mRNA expression (data not shown). Taken together, these data demonstrate that reactivation of MCT3 expression by 5-aza-dC may be a direct effect of demethylation, further supporting the causative effect of DNA methylation in MCT3...
repression in dedifferentiated SMCs. The lack of a further increase in MCT3 expression in response to 5-aza-C in differentiated SMCs and RPE indicates that methylation of scattered CpGs in the promoter region of these cells makes little, if any, contributions to suppression of the gene.

Impaired Lactate Transport and Enhanced Proliferation in Cultured SMCs

To determine whether MCT3 silencing, probably controlled by DNA methylation, resulted in impaired lactate transport, we determined intracellular acidification induced by extracellular application of lactate with carboxysemianaphorodofluor-1 (carboxy-SNARF-1) as an indicator for pH, and used confocal microscopic analysis in 5 primary SMC strains incubated in the presence or absence of lactate. SNARF staining of SMCs at passages 1 and 8 incubated in serum-free Dulbecco’s modified Eagle’s medium in the absence of lactate for 2 hours revealed essentially the same basal level of pH (Figure 7A–7D). After a 2-hour incubation with 25 or 50 mmol/L lactate, SMCs at passage 1 exhibited a marked and significant decrease in pH, whereas SMCs at passage 8 did not show detectable changes (Figure 7E–7H). To confirm that the observed acidification reflected the entry of lactate and H\(^+\) via MCTs, we pretreated the cells with phloretin, an inhibitor of MCTs. Phloretin treatment abolished the pH decrease in SMCs at passage 1 but elicited little if any change in SMCs at passage 8 (Figure 7I–7L). Because phloretin also inhibits glucose transport mediated by sodium-independent glucose transporter-1 and -4 and therefore might affect lactate transport, we repeated the aforementioned experiments with siRNA-mediated MCT3 gene silencing. Selective MCT3 knockdown similarly eliminated the pH decrease in SMCs at passage 1, but not passage 8, whereas scrambled siRNA controls had no effect (data not shown).

If reexpressed MCT3 induced by demethylation is functional, we reasoned then that lactate transport might be restored in passage 8 SMCs after 5-aza-dC treatment. Indeed, confocal microscopic analysis revealed a decrease in pH in these cells after lactate incubation equivalent to that seen in passage 1 SMCs. In contrast, 5-aza-dC treatment did not induce a detectable change in the capacity for lactate transport in passage 1 cells (Figure 7M–7P). These data, in conjunction with the MCT3 reactivation results, indicate that lactate transport in contractile SMCs is mediated by MCT3, which is further modulated by DNA methylation.

To investigate the significance of MCT3 methylation in affecting SMC function, we treated passage 2 SMCs, prolifer-
ating SMCs that still express high levels of MCT3, with 4 MCT3-specific and 1 scrambled siRNA control oligonucleotides. As shown in Figure 8, MCT3-specific siRNA markedly and dose-dependently reduced MCT3 mRNA and protein expression and stimulated SMC proliferation, whereas the scrambled siRNA control had no effect. These data indicate that MCT3 inhibition and the subsequently impaired lactate transport and intracellular acidification may result in enhanced SMC proliferation, contributing to neointimal hyperplasia.

**Discussion**

Atherosclerosis and cancers share many similarities in their pathogenesis. For example, they are susceptible to the same set of risk factors, including aging and smoking, and involve aberrant cell proliferation in their disease processes. Interestingly, global DNA hypomethylation and locus-specific hypermethylation, which have received considerable attention in the cancer research field, have been inadequately studied in relation to atherosclerosis. Indeed, it is conceivable that DNA methylation might play a more predominant role in the pathogenesis of atherosclerosis, in that, unlike oncogenesis, which has been linked to base-pair change, deletion, insertion, recombination, and amplification of oncogenes (genomitis), atherogenesis involves mainly changes in gene function rather than structural mutations. The functional changes may result from alterations in gene expression due to polymorphisms, most commonly single-nucleotide polymorphisms (SNPs) and differential epigenetic changes, in particular, DNA methylation. Although completion of human genome sequencing has led to global gene expression profiling and SNP searching becoming busy industries, epigenetic research has not gathered momentum in the field of atherosclerosis.

The findings presented in this report underscore the importance of DNA methylation in atherogenesis, suggesting that DNA methylation may represent an important process whereby atherosclerosis risk factors affect the function of the genome, a responsive organ of the cell, and induce atherosclerotic changes. Our data also shed light on understanding of the regulation of monocarboxylate transport in the vessel wall, a previously unrecognized biological aspect, in particular with respect to atherosclerosis.

MCT3, originally cloned from a chicken RPE expression library, is thought to be preferentially expressed in the RPE. Using multiple approaches including quantitative TRT-PCR and Western blotting, we have unequivocally demonstrated the expression of MCT3 mRNA and protein in normal tunica media of the vessel wall. These data provide evidence, for the first time, of MCT3 expression in an extra-RPE tissue. Furthermore, MCT3 exhibited a passage-dependent decrease in cultured SMCs and a lesion-dependent decrease in atherosclerotic vessels. Immunohistochemical staining revealed that the lack of MCT3 expression was restricted to proliferating/synthetic SMCs residing in the neointima in atherosclerotic coronary arteries, indicating that the SMC phenotypic switch may serve as a trigger for MCT3 silencing. These findings provide a basis for the use of the tunica media and cultured SMCs as proxies for SMC expressers and nonexpressers, respectively, to investigate the mechanisms that control MCT3 expression, as described in this study.

DNA methylation has emerged as an important epigenetic modification that regulates gene transcription. In general, DNA methylation represses transcription, and loss of methylation is associated with gene activation. Many previous studies have focused on the gene-silencing effects of methylation of CpG islands located in promoter and exon 1 regions. The conventional wisdom is that DNA methylation in these transcription initiation regions can either directly interfere with transcription factor binding or indirectly via methyl-CpG binding proteins, which can mediate histone modification by recruiting histone deacetylases to methylated DNA. Although it is clear that...
promoter hypermethylation is an important mechanism in suppressing gene expression, recent evidence indicates that DNA methylation in the coding regions also serves as a silencer. Indeed, using patch-methylated stable episomes in human 293 cells, Hsieh and Irvine et al have demonstrated that methylation of the coding region of a luciferase gene represses transcription 5-fold more effectively than does methylation of the Rous sarcoma virus long terminal repeat promoter. Furthermore, methylation impacts transcriptional elongation more than initiation in fungi. By using the Cre/loxP-based system, RMCE, to introduce a transgene methylated exclusively in a region downstream of the promoter into a specific genomic site, Lorincz et al found that dense, intragenic DNA methylation in the coding regions also serves as a promoter hypermethylation is an important mechanism in

observed decrease in MCT3 protein expression, as measured by Western blotting, which is based on equal loading of proteins. SMCs not only produce lactate through glycolysis but also take it up and use it as respiratory fuel. It has long been known that lactate cannot move freely from muscle to blood. Experiments with both in situ and isolated muscles have suggested that the translocation of lactate across the plasma membrane is mediated via the saturable, stereospecific MCT transport system that exhibits an obligatory 1:1 coupling between lactate and H+ By measuring the rate of decline in pH, after addition of 25 or 50 mmol/L lactate, which predominantly reflects the entry of lactate and H+ via the MCT transport system, the reverse of its normal physiological function when lactic acid derived from anaerobic metabolism is extruded from the cell, we found that monocarboxylate transport activity in SMCs at passage 1 was exclusively mediated via MCTs, because phloretin, an inhibitor of MCTs, and MCT3 siRNA completely abolished acidification after lactate incubation. The absence of monocarboxylate transport activity in SMCs at passage 8 and restoration of activity in these cells after 5-aza-dC treatment, coupled with the silencing and reactivation of MCT3 expression before and after 5-aza-dC incubation in these cells, respectively, point to a central role for MCT3 in SMCs, in particular, the differentiated tunica media. The lack of compensatory changes in MCT1, 2, and 4 expression and the inhibition of lactate transport by siRNA-mediated specific MCT3 knockdown lend further support to a key role for MCT3 in regulating monocarboxylate transport. Importantly, suppression of MCT3 expression by siRNA resulted in markedly enhanced proliferation of SMCs, which links MCT3 methylation and silencing to neointimal hyperplasia in atherosclerosis.

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