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Hypomethylation of DNA: A Possible Nongenotoxic Mechanism Underlying the Role of Cell Proliferation in Carcinogenesis

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DNA methylation (i.e., the 5-methylcytosin content of DNA) plays a role in the regulation of gene activity. There is a persuasive body of evidence indicating that differential methylation of DNA (i.e., 5-methylcytosine versus cytosine) is a determinant of chromatin structure and that the methyl group provides a chemical signal that is recognized by trans-acting factors that regulate transcription. Hypomethylation (i.e., low levels of DNA 5-methylcytosine) of a gene is necessary but not sufficient for its expression, and, therefore, a hypomethylated gene can be considered to possess an increased potential for expression as compared to a hypermethylated gene. Cell proliferation is a fundamental component of carcinogenesis. It plays a key role in expanding clones of initiated cells and, in addition, cell replication may contribute to carcinogenesis by facilitating mutagenesis. This can occur either by causing the fixation of promutagenic DNA-damage before repair or as a consequence of a "normal" error occurring during DNA replication. During periods of cell proliferation the established pattern of DNA methylation is maintained by the action of a maintenance methylase following DNA replication. Changes in the methylation status of a gene provide a mechanism by which its potential for expression can be altered in an epigenetic heritable manner, and it is expected that modifications in DNA methylation would result from threshold-exhibiting events.

Introduction

A decrease in DNA-5-methylcytosine (i.e., hypomethylation) may facilitate the aberrant expression of protooncogenes involved in carcinogenesis. We are proceeding to test our hypothesis that hypomethylation of DNA is a nongenotoxic mechanism underlying the role of cell proliferation in carcinogenesis. Clearly, mutagenesis appears to be involved in carcinogenesis. However, with the possible exception of tumor-suppressor genes, a mutated gene must be expressed to affect the phenotype of a cell. Therefore, our hypothesis is fully compatible with, and complementary to, the notions of Cohen and Ellwein (*11*) and Ames and Gold, (*12*) regarding the role of cell proliferation in carcinogenesis.

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Epigenetics and Consideration of Inheritance on a Dual Level

Currently, we recognize the fact that there is a need to consider inheritance on a dual level. That is, to distinguish the transmission of genes from generation to generation (i.e., inheritance of DNA base sequence) from the mechanisms involved in the transmission of alternative states of gene activity in somatic cells. "Epigenetics" is the term used to describe the latter, and it may be defined as the study of mechanisms responsible for the temporal and spatial control of gene activity, e.g., changes in gene expression during development, segregation of gene activities such that daughters of an individual cell have different patterns of gene expression, and mechanisms to permit the somatic inheritance of a specific set of active and quiescent genes (*1*). DNA methylation is one epigenetic mechanism by which gene activity may be regulated.

DNA Methylation and Regulation of Gene Activity

DNA methylation (i.e., the 5-methylcytosine content of DNA) plays a role in the regulation of gene activity

(*2*). There is a persuasive body of evidence indicating that differential methylation of DNA (i.e., 5-methylcytosine versus cytosine) is a determinant of higher-order chromatin structure (*3*) and that the methyl group provides a chemical signal that is recognized by trans-acting factors. Binding or lack of binding of these factors prevent transcription (*1*). Thus, DNA methylation appears to be a mechanism whereby cells can control the expression of genes with similar promoter regions in the presence of ubiquitous transcription factors (*4*). Hypomethylation of a gene, i.e., low levels of DNA 5-methylcytosine (DNA-5MeC), is necessary but not sufficient for its expression. Therefore, a hypomethylated gene can be considered to possess an increased potential for expression as compared to a hypermethylated gene (*5*).

While methylation in the vicinity of the 5' end of a gene often appears to be involved in the regulation of its transcription, hypomethylation in the middle and in the vicinity of the 3' end have also been correlated with gene expression (*6*). This is illustrated in an elegant series of studies in which it was demonstrated that the capacity of a plasmid containing the human Ha-*ras* oncogene, mutated by a G to T transversion at codon 12, to transform NIH-3T3 cells was reduced 80% after methylation *in vitro* at all of its CCGG and GCGC sites by the methyl transferases HpaII and HhaI, respectively. Treatment of the plasmid with either of the methyltransferases alone had no effect (*7*). In addition, the influence of methylation on gene expression was assessed using an experimental system consisting of a plasmid containing the chloramphenicol acetyltransferase (CAT) gene linked to either the Ha-*ras* gene promoter region, which is enriched with CpG islands, or a promoter consisting of the Rous sarcoma virus (RSV) long terminal repeat (LTR), which does not contain CpG islands (*8*). Methylation of the plasmids *in vitro* with methyltransferases HpaII plus HhaI and with human placental methyltransferase resulted in marked inhibition of expression. CAT expression was decreased approximately 50% in those experiments in which the LTR-containing plasmid was employed, while expression was reduced >95% when the Ha-*ras* gene promoter-containing plasmid was used (*8*). Taken together, these studies indicate that regulation of gene expression can involve multiple methylatable cytosine-containing sites in both the promoter region and in regions downstream of the transcription start site and that a high percentage of these sites might have to be methylated to render the gene completely quiescent. This is consistent with recent observations indicating that the binding of a repressor protein is correlated directly with the density and distribution of methylated CpG sites (*9*).

Cell Proliferation, DNA Methylation, and Carcinogenesis

In vivo, 5-methylcytosine is most often found in DNA at CpG residues, and each site is actually modified on both strands (*2*), i.e., DNA methylation is symmetrical. During periods of cell proliferation, the established pattern of DNA methylation is maintained by the action of an *S*-adenosylmethionine-requiring maintenance methylase. Immediately after DNA replication, the newly synthesized strand is unmodified (i.e., not methylated). However, the maintenance methylase is specific for hemimethylated sites (*10*), and, therefore, it can maintain the normal symmetrical pattern of DNA methylation. Thus, a CpG site that is initially unmodified can remain that way after replication, while a methylated CpG site will be recognized by the methyl group remaining on the parental strand and be methylated on the complementary strand (*2*). Changes in the methylation status of a gene provide a mechanism by which its potential for expression can be altered in an epigenetic heritable manner (*1,4*) and, in light of the steps involved in DNA maintenance methylation, it is expected that modifications in DNA methylation would result from threshold-exhibiting events.

Cell proliferation is a fundamental component of the multistage process of carcinogenesis (*11*). It plays a key role in expanding clones of initiated cells, and, in addition, cell replication may contribute to carcinogenesis by facilitating mutagenesis (*12*). This can occur either by causing the fixation of promutagenic DNA damage before repair or as a consequence of a "normal" error occurring during DNA replication. In addition, during periods of cell replication, and especially in an animal chronically exposed to the maximum tolerated dose of a test compound, there is the possibility for heritable decreases in DNA-5-MeC (i.e., hypomethylation) due to a limitation in the capacity for and/or fidelity of DNA maintenance methylation. This is expected to be a threshold-exhibiting event and could result in a heritable, epigenetic, increase in the potential for gene expression.

We do not anticipate a simple one -to -one relationship between the level of cell proliferation and hypomethylation of DNA. The ability to maintain the nascent pattern of methylation is dependent on a complex relationship between the capacity and fidelity of DNA maintenance methylase, the amount of S-adenosylmethionine, and the level of cell proliferation.

Evidence for hypomethylation and cell replication playing a role in carcinogenesis comes from studies involving the use of a choline-devoid (CD) diet. Fischer-344 rats (*13,14*) and B6C3F$_1$ mice (*15*) develop liver tumors, even without exposure to chemical carcinogens, after they are fed a CD diet. Dietary choline and methionine are required to maintain hepatic intracellular levels of *S*-adenosylmethionine, and a depletion of this cofactor for methylation reactions might lead to hypomethylation of DNA with concomitant alterations in the expression of proto-oncogenes, including Ha-*ras* (*16*). Alterations in DNA methylation have been suggested to play a role in carcinogenesis (*17*). Furthermore, increased liver cell proliferation, as

measured by incorporation of ^{3}H-[methyl]-thymidine into DNA and an increase in the percent of hepatocytes in mitosis, can be detected within 1 week after placing rats on a CD diet (*18*). This increased proliferation of hepatocytes was sustained during a 9-week period while the animals were maintained on the CD diet. Thus, a CD diet-induced decrease in *S*-adenosylmethionine levels in the presence of increased cell proliferation may result in hypomethylation of DNA.

Hypomethylation of DNA may facilitate the aberrant expression of proto-oncogenes involved in carcinogenesis. We are proceeding to test our hypothesis that hypomethylation of DNA is one of the mechanisms underlying the role of cell proliferation in carcinogenesis (*5,19*). Clearly, mutagenesis appears to be involved in carcinogenesis. However, with the possible exception of tumor-suppressor genes, a mutated gene must be expressed to affect the phenotype of a cell. Therefore, our hypothesis is fully compatible with, and complementary to, the notions of Cohen and Ellwein (*11*) and Ames and Gold (*12*) regarding the role of cell proliferation in carcinogenesis.

Hypomethylation of DNA in Mouse Liver Tumors

The liver tumor-prone B6C3F$_1$ mouse (C57BL/6♀ × C3H/He♂), in conjunction with the more susceptible C3H/He paternal strain and relatively resistant C57BL/6 maternal strain, provides an excellent model for studying mechanisms involved in carcinogenesis (*19*). A relative decreased capacity for and/or fidelity of DNA maintenance methylation, in addition to a lower nascent level of methylation, might contribute to the high susceptibility to liver tumorigenesis exhibited by certain strains of mice (e.g., C3H/He and B6C3F$_1$). The presence of activated oncogenes has been detected in both spontaneous and chemically induced B6C3F$_1$ mouse liver tumors, but not in normal liver. Further examination has revealed that Ha-*ras* is involved in approximately one-third to one-half of these tumors. However, a critical mutation in the Ha-*ras* gene per se cannot be the heritable characteristic that predisposes the B6C3F$_1$ mouse to development of liver tumors because nascent liver tissue does not possess this alteration.

Ha-*ras* in the liver of B6C3F$_1$ and C3H/He mice lacks a methylated site (5'-MeCCCG-3') that is present in the gene in C57BL/6 liver, and hepatic Ha-*ras* appears to possess an additional DNAse I hypersensitive site in the B6C3F$_1$ and C3H/He strains as compared to the C57BL/6 strain (*19*). This indicates an increased potential for expression of Ha-*ras* in the hepatoma-prone strains which could, in part, account for high propensity for liver tumor development in these strains. In addition, this might account for the high frequency with which an activated Ha-*ras* is associated with B6C3F$_1$ and C3H/He mouse liver tumors. Furthermore, Ha-*ras* has been found to be hypomethylated in B6C3F$_1$ mouse liver tumors induced by a genotoxic compound (benzidine) or nongenotoxic compounds (phenobarbital [PB] or chloroform) as well as in spontaneous tumors (*19*), indicating that hypomethylation might be a common event involved in oncogene activation.

The *raf* protooncogene encodes a serine-threonine kinase implicated in signal transduction downstream of, or independent of, Ha-*ras* (*20*). Elevated levels of *raf* mRNA have been detected in rat liver tumors (*21*), and rodent liver epithelial cells transfected with v-*raf* exhibit a transformed phenotype (*22*). Therefore, *raf* is a reasonable candidate for involvement in mouse liver tumorigenesis, either in conjunction with Ha-*ras* or in those situations when an activated Ha-*ras* gene is not detected.

The methylation status of *raf* was assessed by restriction of mouse liver DNA with methylation-sensitive isochizomers and Southern blot analysis. An *Mspl* restriction fragment length polymorphism was seen as the presence of a 6.7 kb band in the hepatoma-resistant C57BL/6 strain but not in the hepatoma-prone C3H/He strain. This band is due to a specific 5'-MeCCGG-3' site in *raf* in the resistant strain that is not present in the hepatoma-prone strain, and this is similar to our findings noted above for Ha-*ras*. The hepatoma-prone B6C3F$_1$ (C57BL/6♀ x C3H/He♂) mouse inherits a raf allele from the C57BL/6 which contains that 5'-MeCCGG-3' site. This site is unmethylated in B6C3F$_1$ liver tumors induced by PB (150 mg/kg/day for 2 years). However, the methylation status of a 5'-CMeCGG-3' site in exon 12 was not altered. These data, the first to suggest a role for *raf* in mouse liver tumorigenesis, indicate that a selective hypomethylation of *raf* is associated with PB-induced mouse liver tumorigenesis and that this might facilitate aberrant expression of the gene (*23*).

Conclusion

Taken together, the investigations discussed here support our hypothesis that hypomethylation of DNA is a nongenotoxic mechanism underlying the role of cell proliferation in carcinogenesis. This could facilitate the aberrant expression of oncogenes that is believed to play a role in the multistep process by which a normal cell is transformed into frank malignancy. In recent years it has become apparent that it is increasingly difficult to live with a bioassay-driven definition of a carcinogen (*11*), i.e., a carcinogen is simply a chemical that increases the incidence of tumors in a given tissue in a given period of time in a bioassay. The answer to the question of whether a chemical might act as a carcinogen depends on a variety of factors, including dose and the test animal used. A rational approach toward assessment of the carcinogenic potential that a chemical might pose requires the incorporation of biological information (*24*). The testing of the hypothesis we have presented and discussed here offers the potential to provide the type of mechanistic insight required.

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