Inflammation-Mediated Cytosine Damage: A Mechanistic Link between Inflammation and the Epigenetic Alterations in Human Cancers

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Abstract

Aberrant methylation patterns have long been known to exist in the promoter regions of key regulatory genes in the DNA of tumor cells. However, the mechanisms by which these methylation patterns become altered during the transformation of normal cells to tumor cells have remained elusive. We have recently shown in *in vitro* studies that inflammation-mediated halogenated cytosine damage products can mimic 5-methylcytosine in directing enzymatic DNA methylation and in enhancing the binding of methyl-binding proteins whereas certain oxidative damage products inhibit both. We have therefore proposed that cytosine damage products could potentially interfere with normal epigenetic control by altering DNA-protein interactions critical for gene regulation and the heritable transmission of methylation patterns. These inflammation-mediated cytosine damage products may provide, in some cases, a mechanistic link between inflammation and cancer. [Cancer Res 2007;67(12):5583–6]

Cytosine methylation patterns in human cells are now recognized as important elements of cellular coordination of gene expression. Cytosine methylation in eukaryotes is a postreplicative enzymatic process. Methylation occurs predominantly in CpG dinucleotides in islands of concentrated CpG sequences located within promoter regions. Methylation of CpG islands in promoter regions is generally associated with gene silencing, whereas absence of cytosine methylation is correlated with transcription (1). Much of the evidence in support of the role of methylation in gene silencing comes from studies using deoxycytidine analogues, 5-azacytidine and zebularine, which interfere with enzymatic DNA methylation of cytosine residues, resulting in hypomethylation and reactivation of transcription.

The mechanisms by which cytosine methylation could be involved in epigenetic regulation have been revealed by several *in vitro* studies. It is known that symmetrical methylation of a CpG dinucleotide can increase the binding of proteins that recognize methylated CpG dinucleotides, the methyl-binding proteins, by a factor of 100 (2). The binding of methyl-binding proteins is thought to be an initial event in the cascade of DNA protein interactions that lead to chromatin condensation and gene inactivation (3). The methyl-binding proteins also possess domains that interact specifically with histone modification enzymes, suggesting a pathway by which DNA methylation and histone modification might be linked. The interplay between cytosine methylation and histone modification in the control of gene activity has yet to be completely understood. It is not clear whether histone modification drives cytosine methylation changes or vice versa. However, it is clear that methylation changes are associated with transcription modification under both physiologic and pathologic conditions. Altering cytosine methylation within a cell can potentially perturb a multitude of DNA-protein interactions that normally allow for controlled expression of tumor promoter genes and repression of tumor suppressor genes.

The faithful heritability of cytosine methylation patterns from parent to progeny in somatic cells is essential to the epigenetic control of gene expression. Aberrant expression or silencing of genes within progeny cells due to even temporary relaxation of the fidelity of the maintenance methyltransferase can be functionally equivalent to polymerase miscoding and mutation in terms of causing permanent alterations. *In vitro* studies with purified DNA methyltransferases have revealed mechanistically how cytosine methylation patterns can remain true from parent to progeny throughout multiple rounds of cell division. Following DNA replication, the symmetrically methylated CpG dinucleotide is converted to a hemimethylated substrate due to semiconservative DNA replication. The hemimethylated CpG sequence is the preferred substrate for the human maintenance methyltransferase, DNMT1, as the methyl group on the parental 5-methylcytosine directs maintenance methylation of the target cytosine in the daughter strand (4). Specific methylation of these hemimethylated sites following DNA replication then allows for the heritable transmission of methylation patterns in differentiated cells.

Because DNA methylation is a critical player in orchestrating the epigenetic control of gene expression, it is not surprising that alterations or perturbations of methylation patterns have been implicated in the development of human cancer. Indeed, a substantial and growing list of human genes display altered methylation status in human tumors (5). Two general events are known to occur in the alteration of methylation patterns seen in human tumors: (*a*) aberrant loss of cytosine methylation with the inappropriate expression of transforming genes, and (*b*) aberrant gain of cytosine methylation with the corresponding silencing of tumor suppressor genes. The observation of altered methylation patterns in tumor cells has led to diagnostic as well as prognostic tests for certain human cancers (6).

It is thought that methylation changes, with resulting changes in gene activity, might actually drive tumorigenesis. Research efforts at the level of clinical trials have also been made toward epigenetic targets in chemotherapy as well as chemoprevention of cancer (7). The majority of these agents target the methyltransferases to reverse the propagation of observed aberrant hypermethylation. Although many studies have catalogued methylation changes in a

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variety of human tumor types, and progress is continuing toward improved diagnostic and therapeutic approaches for cancers known to result from methylation changes, very little is known about the events responsible for these changes. A mechanistic understanding of these events will likely hasten the development of both diagnostic and therapeutic methods in cancers where aberrant methylation is implicated as a critical genomic insult. Furthermore, understanding the mechanism by which methylation is perturbed will lead to a more targeted approach for chemoprevention of epigenetic changes that might drive cancer development.

Our group has investigated relationships between DNA damage and methylation changes that reveal intriguing potential connections and expand the role of DNA damage in cancer development to include epigenetic changes in addition to genetic mutations. Unrepaired DNA damage has long been thought to contribute to carcinogenesis by polymerase-directed miscoding and mutation. Our investigations suggest that the role of DNA damage in tumor development may not be limited to mutagenesis. In accord with others, our laboratory has shown that many forms of DNA damage inhibit critical DNA-protein interactions. We show that certain cytosine damage products inhibit both the binding of methyl-binding proteins and DNMT1-mediated methylation of the target cytosine in CpG dinucleotides (2, 8). Interrupting the cascade of events important in gene silencing via interference with binding of methyl-binding protein and DNMT1 methylation could lead to the inappropriate activation of genes involved in cell proliferation. Viewed from a structural standpoint, most forms of cytosine damage would likely interfere with both protein binding and DNMT1 methylation activity due to interference with the critical contact points that define these specific DNA-protein interactions.

Alternatively, a much smaller group of cytosine damage products would be expected to facilitate both binding of methyl-binding protein and enzymatic methylation. Our studies have revealed one form of DNA damage that may prove particularly important in this context: halogenated cytosine residues. Numerous previous studies have shown that halogen atoms, particularly bromine, can substitute for the methyl group of thymine in DNA-protein interactions (9, 10). Whereas these earlier studies examined halogenated analogues with the primary aim of probing DNA-protein interactions, recent evidence suggests that halogenation of nucleic acids can be a significant form of DNA damage in living organisms. Specifically, in areas of tissue inflammation, HOCl from activated neutrophils and HOBr from activated eosinophils are known to react with DNA to form, among other products, 5-chlorocytosine and 5-bromocytosine (11–13). These inflammation damage products have been detected in human leukocytes without exogenous HOCl exposure and are thought to be due to endogenous processes (13). Our studies have established a potential role for these halogenated cytosine damage products in methylation changes, which may provide insight into the connection between inflammation and cancer.

We were intrigued to see if halogenated cytosine residues could mimic methylation in DNA-protein interactions involved in DNA methylation pathways. We therefore constructed halocytosine-containing oligonucleotides and examined the binding of methyl-binding proteins. We observed that the methyl-binding proteins cannot distinguish methylated and halogenated DNA, binding to both with high affinity. We then tested whether this mimicry would also occur with DNMT1-mediated methylation. Our studies revealed that DNMT1 could indeed be similarly fooled by halogenated cytosine; it cannot distinguish between 5-methylcytosine and certain 5-halocytosine residues. We observed that the halocytosines were nearly as good as, and in some cases even better than, 5-methylcytosine in directing DNA methylation of the target cytosine in the CpG sequence context (14). It seems that the size and hydrophobic properties of chlorine and bromine substituents most closely mimic the methyl group of 5-methylcytosine (Fig. 1). The formation and persistence of 5-halocytosine residues in CpG dinucleotides in the DNA of cells at sites of inflammation would be expected to lead to inappropriate *de novo* methylation.

We have searched for repair activity against halogenated cytosine residues in nuclear extracts. We have also examined known pyrimidine glycosylases including Mug and methyl-CpG binding domain protein 4 for activity against halocytosines, but we have found little activity against 5-bromocytosine and no activity against 5-chlorocytosine (14, 15). Indeed, if 5-chlorocytosine and 5-bromocytosine residues are recognized as 5-methylcytosine residues by methyl-binding proteins and DNMT1, it is perhaps not surprising that they are not recognized as damaged bases by DNA repair glycosylases. Failure to recognize and repair 5-chlorocytosine and 5-bromocytosine damage products could lead to the accumulation of these analogues within the genome.

The methyl group on 5-methylcytosine is central to methylation-mediated epigenetic control. The methyl group of 5-methylcytosine is a beacon for critical DNA-protein interactions. Located in the major groove of a B-form DNA helix, the methyl group contributes substantially to the affinity of specific protein-DNA binding. Indeed, the selectivity of the binding of methyl-binding protein to methylated CpG dinucleotides can be attributed to favorable interactions with the methyl group, the magnitude of which approaches a theoretical maximum (16). The biological importance of the methyl group of 5-methylcytosine is further revealed by the existence of cellular mechanisms that prevent 5-substituted cytosine analogues from being metabolized to the triphosphate for utilization by DNA polymerase. Whereas thymidine analogues with 5-substituents are metabolized and incorporated into DNA, the corresponding deoxycytidine analogues are poorly incorporated. The 2′ deoxycytidine analogues with substituents larger than hydrogen are good substrates for deaminases but weak substrates for kinases, reducing their incorporation into DNA (17, 18).

Halogenated cytosine damage products may represent an important link between inflammation and cancer development, two areas long associated through epidemiologic studies. Initial halogenation could assist in the binding of methyl-binding proteins, allowing for facilitated recruitment of histone-modifying enzymes, triggering the cascade of events that result in gene silencing. Perhaps even more profoundly, this initial halogenation of cytosine could direct methylation of the complementary DNA strand, resulting in heritable alterations in methylations patterns.

Inflammation has long been associated with certain cancers. Patients afflicted with inflammatory bowel diseases such as Crohn's disease and ulcerative colitis are at increased risk of the development of colon cancer as compared with the nonafflicted population (19). Based on evidence from epidemiology, genetics, molecular biology, and histopathology, it is also thought that pathogenesis of prostate cancer can also be driven by inflammation (20). An increase in promoter methylation of genes important in minimizing oxidative damage to the genome, including the promoter of the GSTPi gene, has been shown to be hypermethylated and silenced in virtually all prostate cancers (21). Aberrant silencing of tumor suppressor genes is thought to be an initial event in the development of prostate tumors (21).

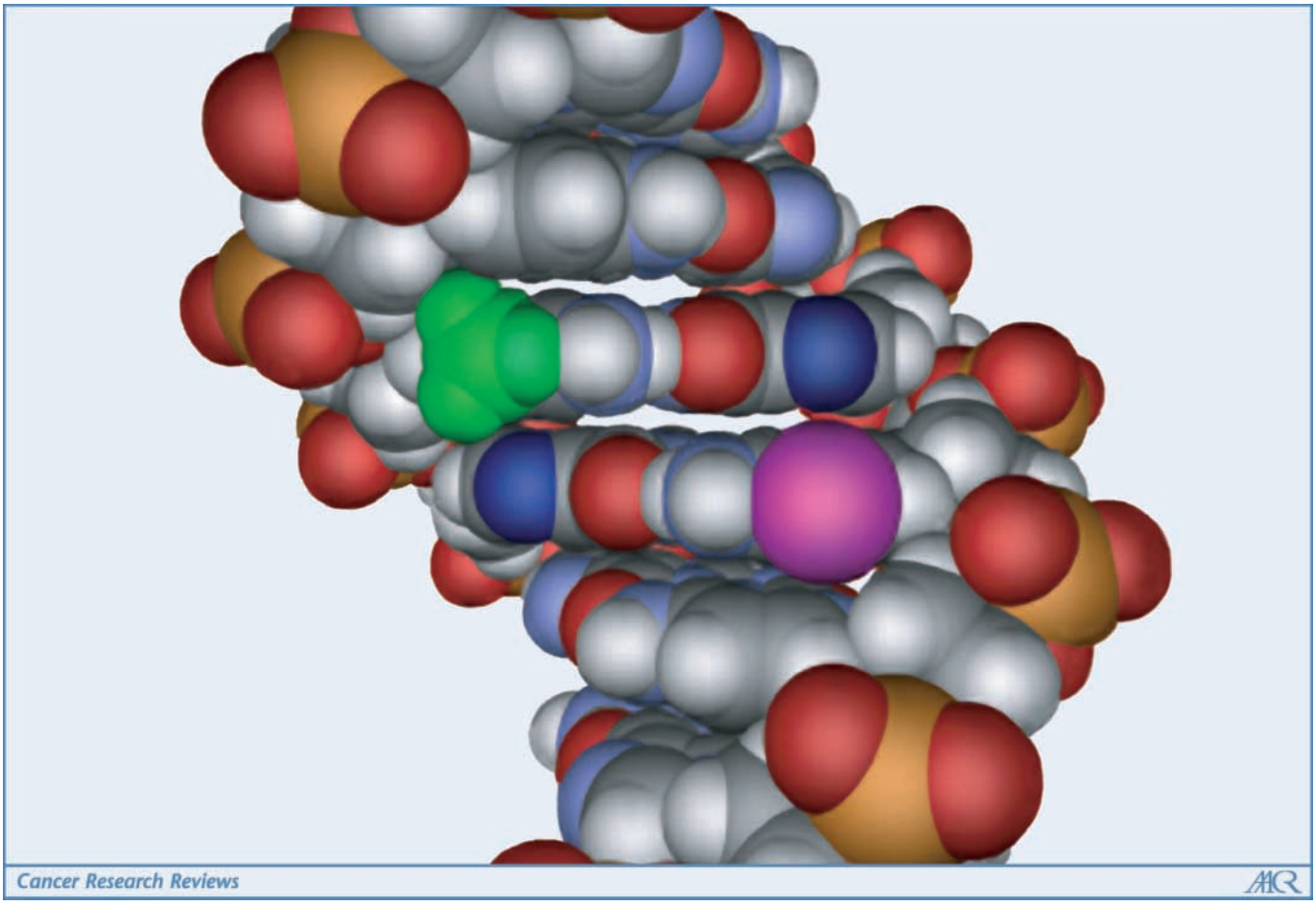

Figure 1. Molecular model of the sequence 5′-GC5mCGGC-3′/3′-CGGClCCG-5′. *Green,* methyl group of 5-methylcytosine; *magenta,* chlorine atom of 5-chlorocytosine. Replacement of the methyl group with a chlorine atom results in a contact point with nearly identical dimensions and positioning of the methyl group. This explains the high affinity of methyl-CpG binding domain for 5-chlorocytosine and 5-bromocytosine halogenated damage products as well as the ability of these 5-halogenated cytosine damage products to mimic 5-methylcytosine in directing maintenance methylation by DNMT1. Halogenated cytosine damage products have the ability to mimic endogenous methylation, likely providing a mechanistic link between the long associated inflammation and cancer.

We believe that our studies provide a mechanistic explanation for some associations between inflammation and methylation changes seen in many human cancers. These data could, in part, rationalize the notion that the pathogenesis of certain cancers is driven by inflammation. It is likely that the DNA damage resulting from attack of inflammation-mediated reactive species could potentially lead to the heritable changes in gene expression as is observed in human cancer. The next step in dissecting this potential pathway of inflammation-mediated cancer development is through identification of specific markers of inflammation that could be associated with increased levels of inappropriate methylation.

Although associations between aberrant methylation patterns and tumorigenesis have been made, the mechanisms by which the aberrant patterns arose are only just beginning to be uncovered. Inflammation-mediated DNA damage products are now reasonable targets for future studies. Confirmation of our results *in vivo*, however, will be difficult, owing to the multitude of DNA damage products resulting from the treatment of cells with chemical agents or activated inflammatory cells. Our studies indicate that some damage products will inhibit methylation, whereas a limited subset might promote aberrant methylation, suggesting that the interpretation of *in vivo* results will be difficult. Nevertheless, studies *in vivo* to address this hypothesis are warranted and should be pursued.

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