DNA Methylation Errors and Cancer

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DNA Methylation and Cancer

It has become clear over the last few years that DNA methylation is essential for normal embryonic development and that alterations in DNA methylation are very common in cancer cells and are capable of directly modifying carcinogenesis. Cytosine methylation is responsible for the induction of a surprisingly high percentage of disease-causing point mutations in tumor suppressor genes in somatic and germline cells. This may either be due to the spontaneous deamination of 5-methylcytosine or to a more active process involving side reactions during the enzymatic modification of cytosine in DNA. Current interest in the role of methylation has focused on the potential for abnormal methylation events to silence tumor suppressor genes, thus giving rise to a novel pathway to cause their progressive epigenetic inactivation. Random methylation errors resulting in the de novo methylation of CpG islands not methylated in normal cells may contribute to the progressive inactivation of growth-inhibitory genes resulting in the clonal selection of cells with increasingly abnormal methylation patterns. This model for gene inactivation during cancer development has important clinical implications, since it is possible to reactivate these dormant genes using inhibitors of DNA methylation and potentially restore growth control to cells.

Mechanism of Action of DNA Methyltransferase

The fundamental chemistry of the methylation reaction was first proposed by Santi et al. (1), who suggested that the addition of the methyl group from S-adenosylmethionine required the formation of a covalent bond between a cysteine residue in the enzyme and the 6-position of the cytosine ring. This mechanism of action has been validated in a variety of experimental approaches, such as the demonstration that incubation of target DNAs with methylases in the absence of the cofactor leads to hydrogen ion exchange on the 5-position of cytosine (2) and to the labilization of the amino group on cytosine of the target DNAs have been resolved recently (7, 8). These elegant studies have shown that the enzymes actually flip the target cytosine about 180° out of the axis of the relatively intact DNA helix into a cleft in the enzyme in which the methyl group transfer is performed. The 5-mCyt ring is then reinserted into the helix, a mechanism which was unexpected, yet resolved questions relating to the steric requirements for methyl group transfer. Base flipping has also been observed for uracil DNA glycosylase (9), and Roberts (10) has suggested that it might represent a general mechanism by which a variety of enzymes interact with DNA. It remains to be shown directly whether base flipping occurs with mammalian methyltransferases. However, the very high degree of evolutionary conservation of amino acids in the catalytic component of the protein strongly suggests that this will be the case.

CpG Islands

Organisms which methylate their genomes have to cope with the increased mutational pressure exerted by this modification. Coulondre et al. (11) were the first to show that cytosine methylation sites in prokaryotes were mutational hotspots and postulated that this was due to the hydrolytic deamination of 5-mCyt. Both cytosine and 5-mCyt deaminate in single- and double-stranded DNA to form uracil and thymine, respectively (12). However, it is inherently more difficult for the cell to correct the resulting T:C mismatch since thymine, unlike uracil, is a normally occurring DNA base and either side of the mismatch can be repaired in principle, giving rise to the correct or incorrect sequence, respectively. Bacteria that have cytosine methylation cope with this problem by expressing very short patch repair systems that correct the premutagenic lesion specifically in the methylated or related sequence context (13, 14). Mammals have evolved a T:G mismatch glycosylase that corrects the T rather than the G (15); however, this system is not altogether efficient and appears to be several hundred-fold slower than uracil glycosylase in human cells (16).

The evolutionary consequences of DNA methylation on the genome have been quite severe (Fig. 1A). Vertebrates and other organisms with DNA methylation show a marked depletion in the frequency of occurrence of the CpG dinucleotide, which is the predominant site at which 5-mCyt is found. The depletion is thought to have been due to increased mutagenesis as described above, and the remaining dispersed CpG sites in the germline and somatic cells are mostly methylated. There are, however, regions of DNA where this suppression has not occurred and these regions, called CpG islands, are often associated with the promoters or coding regions of genes. Importantly, with the exception of those genes and sequences listed in Table 1, CpG islands remain free of methylation in somatic cells. There is a great deal of evidence that methylation of CpG islands results in gene inactivation and that methylated sequences are heritably silenced. As will be discussed below, abnormal methylation of CpG islands is very common in cancer cells and may serve to inactivate some of the genes listed in Table 1.

Methylation in Development and Imprinting

The construction of mice with targeted disruption of the methyltransferase gene has shown that cytosine methylation is essential for normal mammalian development. Mice lacking a fully functional gene die at about mid-gestation (17) and fail to appropriately imprint genes including Igf2, H19, and Igf2r (18). These observations, coupled with those showing parent-specific methylation of CpG island regions

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3 The abbreviations used are: 5-mCyt, 5-methylcytosine; 5-Aza-CdR, 5-aza-2'-deoxycytidine.

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DNA METHYLATION ERRORS AND CANCER

A

Methylation and Mutation

Organisms Without Methylation

Present Day Vertebrates

Mutational Hotspots

B

Growth Regulatory Gene

Random Methylation Errors

Growth Regulator Gene

Clonal Selection of Cells

Further Random Methylation Errors

Silenced Gene

Heterochromatinization

GROWTH↓

GROWTH↑

Fig. 1. A. Evolution of CpG islands. Organisms without cytosine methylation show no suppression in the frequency of occurrence of the CpG methylation site, as indicated in the upper part of the figure. Cytosine methylation (●) in vertebrates and other organisms has led to an approximately 80% suppression in the frequency of occurrence of the CpG site (lower part of figure). The remaining CpG sites are either clustered in 0.5–2-kb regions called CpG islands or are dispersed, in which case they are mostly methylated. These sites are often mutational hotspots and contribute very significantly to the generation of polymorphisms (53), germine mutations (26), and cancer-causing mutations in the p53 and other genes (27). The CpG islands are almost always associated with the promoters (e.g., Ha-ras or HPRT) or coding regions of genes (e.g., MyoD or p16) and are unmethylated on autosomal genes. B, model for the progressive inactivation of tumor suppressor genes by abnormal methylation of CpG islands. Several tumor suppressor genes (Table 1) contain CpG islands which, in common with other autosomal genes, are not normally methylated. Random methylation errors of these CpG sites leads to reduced gene expression, resulting in the clonal selection of cells with these heritable epigenetic defects. Further methylation can result in the eventual paralysis of the gene by heterochromatinization, giving rise to further selection of cells with methylation defects. Note that tumor cells often contain reduced methylation of the dispersed CpG sites, resulting in a decreased overall level of methylation at the same time as focal hypermethylation of CpG islands is observed.
in these genes (Ref. 19–21; Table 1), has firmly established a role for methylation in genomic imprinting. In this regard, it is interesting to note that cancer cells sometimes show loss of imprinting (22), which might be explained by changes in DNA methylation that are known to accompany tumorigenesis.

X-inactivation in female eutherian mammals is a well-characterized developmental event associated with a methylation change in CpG islands. The CpG islands within the promoter regions of the X-linked housekeeping genes become extensively methylated at, or soon after, the onset of this developmental event associated with a methylation change in CpG islands. The CpG islands within the promoter regions of the X-linked genes, the CpG islands associated with autosomal genes remain methylation free, with some exceptions. These exceptions include Alu elements (23) and L1 retrotransposons (24), which are frequently extensively methylated in the human genome. This methylation has been proposed to be part of a host defense system contributing to the silencing of foreign DNA sequences (25).

In summary, methylation of CpG islands on autosomal genes is unusual in normal cells but is a process that leads to heritable inactivation. Although methylation is inherently mutagenic and has led to profound genomic alterations, it is apparently tolerated, possibly because of its necessity during embryonic development.

**Methylation Errors and Mutation**

It has been clear for some time that CpG sites are hotspots for mutation in the human germline (26), and more recently it has become apparent that they are also hotspots for inactivating mutations in tumor suppressor genes (27–29). About 25% of the point mutations in the p53 gene in all human cancers studied occur at CpG sites, and almost 50% occur at methylation sites in colon cancer (30). Since no exogenous chemicals have been shown to increase directly the rate at which these mutations occur (31), they should be considered as part of an endogenous process.

As mentioned earlier, the conventional explanation for the existence of the hotspots has been spontaneous hydrolytic deamination of 5-mCyt to T. However, errors made during the methylation process may also contribute to mutagenesis. DNA methyltransferases can catalyze the deamination of C to U when S-adenosylmethionine is limiting (3) and then, by binding to the resulting U:G mismatch, either shield it from repair or methylate U directly to T (32–34). Recent experiments by Yebra and Bhagwat (35) have shown that cytosine methyltransferases are also capable of the direct conversion of 5-mCyt to T, thus extending the repertoire of side reactions that could contribute to the CpG island sequence methylated (frequency).

**Table 1 Methylation of CpG islands in normal and transformed cells**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>CpG island sequence methylated (frequency)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>• Inactive X-chromosome 43</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Imprinted genes (igf-2, H19, igf-2r) 19, 20, 21</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Alu sequences 23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• L1 sequences 24</td>
<td></td>
</tr>
<tr>
<td>Transformed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retinoblastomas</td>
<td>• Rb (6/77) 54, 55</td>
<td></td>
</tr>
<tr>
<td>Renal cancers</td>
<td>• VHL (5/26) 56</td>
<td></td>
</tr>
<tr>
<td>Various cancers</td>
<td>• p16 (2367) 57, 58, 59</td>
<td></td>
</tr>
<tr>
<td>Bladder cancers</td>
<td>• Myf3 (30) 49</td>
<td></td>
</tr>
<tr>
<td>Cell lines</td>
<td>• E-cadherin (7/11) 60</td>
<td></td>
</tr>
</tbody>
</table>

The pervasive alterations in methylation patterns, particularly those in the abnormal methylation of CpG islands seen in cancer cells, suggest a causative role for altered methylation in carcinogenesis (Fig. 1B). It seems likely that the de novo methylation of CpG islands is not driven by an active wave of methylation, such as that which occurs during embryonic development, but rather arises as a result of chance modification of previously unmethylated palindromes, followed by selection of cells with down-regulated genes. Tumor suppressor genes containing CpG islands (see examples in Table 1) may, therefore, be susceptible to random methylation errors resulting in partial methylation of previously unmethylated CpG islands. This could result in down-regulation of the particular growth-suppressing gene and thus lead to an increased growth rate. As mentioned above, the methylation errors probably result from purely stochastic processes (43) since there is no evidence for the directed de novo methylation of particular sequences during carcinogenesis. The abnormal methylation may result in heritable modification changes and clonal selection of cells with increased growth rates due to decreased suppressor gene activity.

Evidence supporting the idea that methylation changes occur very early in the transformation process comes from studies with immortal but not tumorigenic mouse cell lines. We found that all mouse cell lines tested acquired de novo methylation of the MyoD CpG island during the establishment of immortality (44), and Antequera et al. (45) observed that one-third of the CpG islands in mouse cell lines were abnormally methylated. There is also direct evidence that methylation changes inactivating the estrogen receptor CpG island in apparently normal colon epithelium in older individuals (46). Methylation errors may, therefore, be associated with preneoplastic changes and might be potential targets for prevention strategies.

Methylation Errors and Epigenetic Changes in Cancer

The changes in DNA methylation that accompany carcinogenesis have been summarized in many reviews. Decreases in the overall content of 5-mCyt (37), demethylation of specific loci (38), de novo methylation of CpG islands (39), and increased levels of DNA methyltransferase enzyme (40) have all been observed. These observations collectively have shown that methylation changes are a highly consistent feature of carcinogenesis but have not provided evidence for a causal relationship between the two phenomena.

Recent experiments with mice deficient in methyltransferase have, however, clearly established that decreased methylation caused by genetic or pharmacological manipulation leads to decreased colon polyp formation in mice carrying a predisposing cancer-causing mutation (41, 42). This result provides direct evidence that methylation and carcinogenesis are linked. Other findings strongly supporting a role for aberrant methylation in carcinogenesis come from a series of observations that the CpG islands of growth-regulatory genes, and particularly tumor suppressor genes, are frequently de novo methylated in tumors and cell lines (Table 1). Because of the well-established ability of methylation to silence the expression of CpG islands discussed earlier, these results have the important implication that de novo methylation may be a pathway to the inactivation of tumor suppressor genes.

A Model for Progressive Gene Inactivation by Random Methylation Errors

The pervasive alterations in methylation patterns, particularly those in the abnormal methylation of CpG islands seen in cancer cells, suggest a causative role for altered methylation in carcinogenesis (Fig. 1B). It seems likely that the de novo methylation of CpG islands is not driven by an active wave of methylation, such as that which occurs during embryonic development, but rather arises as a result of chance modification of previously unmethylated palindromes, followed by selection of cells with down-regulated genes. Tumor suppressor genes containing CpG islands (see examples in Table 1) may, therefore, be susceptible to random methylation errors resulting in partial methylation of previously unmethylated CpG islands. This could result in down-regulation of the particular growth-suppressing gene and thus lead to an increased growth rate. As mentioned above, the methylation errors probably result from purely stochastic processes (43) since there is no evidence for the directed de novo methylation of particular sequences during carcinogenesis. The abnormal methylation may result in heritable modification changes and clonal selection of cells with increased growth rates due to decreased suppressor gene activity.

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DNA Methylation as a Therapeutic Target

Several features of the abnormal methylation patterns in tumor cells make them attractive targets for therapeutic intervention: (a) the growth-regulating genes have not been mutated or lost but are present in a suppressed form, which makes them theoretically possible to restore expression and hence reestablish growth control; (b) since few genes are controlled by CpG island methylation in normal somatic cells (Table 1), inhibition of methylation is unlikely to induce promiscuous gene expression in normal cells; and (c) genes silenced by methylation errors are very sensitive to reactivation by inhibitors of DNA methylation such as azacytosine nucleosides (50, 51).

All of the genes listed in Table 1 can have their functions restored (at least in culture) by treatment with azanucleoside analogues. Preliminary evidence in our laboratory has shown that activation of expression of the pl6 tumor suppressor gene might lead to restoration of growth control. The fact that drugs such as 5-Aza-CdR have already been subjected to intense clinical scrutiny (52) and that a great deal is already known about their pharmacokinetics makes it possible that clinical trials to attempt to reactivate genes could begin in the near future.

There are several caveats to these approaches that will require novel strategies for their successful implementation: (a) the approach is inherently a cytotastic one, and the dose of drugs such as 5-aza-CdR will be critical since higher concentrations are not necessarily better in terms of inducing gene expression (50); (b) since there are questions relating to the carcinogenicity and mutagenicity of 5-Aza-CdR, it is unlikely that these drugs could be used for reversion of early-stage methylation errors; and (c) it will be necessary to preselect patients on the basis of demonstrable methylation defects of the target suppressor genes since it will be of little use to treat patients with missing or mutated genes by this modality.

These facts, together with the known chemical instability of 5-aza-nucleosides in aqueous solution, make it unlikely that these drugs will find widespread clinical use, in spite of their potency and specificity for inhibition of DNA methylation. There is, therefore, a need to search for other inhibitors of the DNA methyltransferase. In this regard, it is unlikely that structural isomers of the cofactor 5-adenosylmethionine will be useful since these would be anticipated to inhibit all methylation reactions within the cell.

Conclusion

Changes in the DNA methylation machinery and alterations in DNA methylation patterns and levels are very common in cancer cells. These changes could play a direct role in the induction of point mutations that inactivate tumor suppressor genes. A highly consistent finding in cancer cells is that the CpG islands of growth-regulatory genes are often methylated, in clear contrast to the situation in their normal counterparts. This methylation may be acquired due to random methylation errors resulting in progressive yet potentially reversible loss of gene function.

Acknowledgments

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References


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