Environmental Exposure, DNA Methylation, and Gene Regulation

Lessons from Diethylstilbesterol-Induced Cancers

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ABSTRACT: **DNA methylation is an epigenetic mechanism that regulates chromosomal stability and gene expression. Abnormal DNA methylation patterns have been observed in many types of human tumors, including those of the breast, prostate, colon, thyroid, stomach, uterus, and cervix. We and others have shown that exposure to a wide variety of xenobiotics during critical periods of mammalian development can persistently alter the pattern of DNA methylation, resulting in potentially adverse biological effects such as aberrant gene expression. Thus, this epigenetic mechanism may underlie the observed increased risk in adulthood of several chronic diseases, including cancer, in response to xenobiotic exposures early in life. We present here the lessons learned from studies on the effects of perinatal diethylstilbesterol (DES) exposure on the methylation pattern of the promoters of several estrogen-responsive genes associated with the development of reproductive organs. Perinatal DES exposure, which induces epithelial tumors of the uterus in mice and is associated with several reproductive tract abnormalities and increased vaginal and cervical cancer risk in women, provides a clear example of how estrogenic xenobiotic exposure during a critical period of development can abnormally demethylate DNA sequences during organ development and possibly increase cancer risk later in life. In addition, nutritional factors and stress may also alter DNA methylation during early life and modulate the risk of cancer and other chronic diseases in adulthood. We suggest that DNA methylation status may be influenced by environmental exposures in early life, leading to increased risk of cancer in adulthood.**

KEYWORDS: **methylation; estrogen; diethylstilbesterol; perinatal exposure; carcinogenesis**

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INTRODUCTION: DNA METHYLATION IN DEVELOPMENT AND CANCER

Approximately 3% to 5% of the cytosine residues in mammalian genomic DNA are present as 5-methylcytosine, a result of covalent addition of methyl groups to the cytosine residue.[1] Methylation is catalyzed by DNA methyltansferases, which add methyl groups from S-adenosylmethionines to C5 positions of cytosines. The enzymatic machinery for DNA methylation is composed mainly of three DNA methyltransferases (DNMTs): DNMT1, 3A, and 3B.[2,3] DNMT1, the most abundant DNMT, is constitutively expressed and is required for the maintenance of global methylation after DNA replication. It uses hemimethylated DNA as a preferential template.[4] In contrast, DNMT3 family genes appear to be developmentally regulated and exhibit *de novo* DNA methyltransferase activity *in vitro*. DNMT3 can methylate hemimethylated and unmethylated DNA with equal efficiency.[3]

DNA methylation usually occurs at CpG dinucleotides, which are frequently clustered in regions of about 1–2 kb in length, called *CpG islands*, in or near the promoter and first exon regions of genes.[5–7] DNA methylation is known to silence gene transcription either by preventing/facilitating protein binding or by indirect mechanisms involving changes in chromatin formation. Regulation of DNA methylation leads to control over aspects of development, tissue-specific gene expression, expression of imprinted genes, and silencing of transposable elements.[8] Unmethylated CpG islands are associated with housekeeping genes, while the islands of many tissue-specific genes are methylated or unmethylated, depending on whether they are expressed or not in the tissues.[6,9–11]

DNA methylation plays a key role in mammalian embryonic development, a process that involves differential gene expression or sequentially turning on and off different genes to establish a stable phenotype.[12] Studies of methyltransferase-deficient mice show that mouse embryos expressing low levels of DNMT1 do not develop to term and die at 5 to 20 somite stages, corresponding to the level of the enzymes.[13] DNA methylation is also involved in genomic imprinting, a process in which persistent silencing of a gene from one parent, but not the other, is accomplished.[14] Examples of gene imprinting can be observed in the DNA regions that encode the insulin-like growth factor (IGF)–2 and H19 genes. The H19 gene is maternally expressed and is methylated on the paternal chromosome only. In contrast, the IGF-2 gene is paternally expressed and is methylated on the maternal chromosome only. DNA methylation is also responsible for the inactivation of the X chromosome.[15]

Altered DNA methylation contributes to carcinogenesis as well as to certain developmental disorders.[16] Global hypomethylation is common in cancer tissues as compared to normal tissues.[17] Imbalance of DNA methyltransferase is also frequently observed in tumor tissues.[18,19] Alterations in DNA methylation may contribute to carcinogenesis in several ways, including:

(1) hypomethylation of promoter regions leading to overexpression of oncogenes;
(2) hypermethylation of promoter regions leading to suppression of tumor suppressors;
(3) hypermethylation leading to an increased incidence of deamination of 5-methylcytosine to thymine, leading to C-to-T point mutations in tumor suppressor genes and/or oncogenes.[5]

For example, hypomethylation can be responsible for an increase in expression of oncogenes, as, for example, *c-myc*.[20] Conversely, a variety of tumor suppressor genes can be downregulated or completely silenced owing to promoter region hypermethylation. These include *p16*, *E-cadherin, estrogen receptor*, and the mismatch repair gene *hMLH1*.[21] Alteration of methylation is generally considered an early event in carcinogenesis.[22,23]

DIETHYLSTILBESTROL AND OTHER ENVIRONMENTAL ESTROGENS

Between 1947 and 1971 over 1,000,000 American women were exposed to diethylstilbestrol (DES) when their mothers took the drug during pregnancy to prevent miscarriage. Women exposed to DES during the first three months of pregnancy often exhibited changes in the tissue and/or structure of their uterus, cervix, or vagina. These changes resulted in later infertility problems and also placed them at risk of developing a rare form of cancer, clear-cell adenocarcinoma of the vagina or cervix, at a young age.[24,25] DES also was introduced into the environment because of its estrogenic activity to accelerate growth in cattle. It was estimated that in 1971 alone as much as 27,600 kilograms of DES were used in livestock feed lots.[26]

Several other synthetic and naturally occurring chemicals found in the environment also mimic estrogen. For example, the pesticide 1,1-bis (*p*-chlorophenyl)-2,2,2-trichloroethane (DDT) and some of its congeners are estrogenic, as is the related pesticide methoxychlor. These chemicals are widespread and persistent in the environment. Exposure to these substances occurs throughout life from food, air, water, soil, household products, and probably through breast milk and during development in the mother's womb.[27] In addition, several natural compounds capable of producing estrogenic responses, such as phytoestrogens, also occur in a variety of plants and fungi. Phytoestrogens are widely used as nutritional supplements and nutraceuticals.[28] The human health risks that may be associated with these low-level, yet constant, exposures are still largely unknown and highly controversial.

DNA METHYLATION ALTERATION IN DES-ASSOCIATED ABNORMALITIES AND CANCER

It is well known that treatment of various species, including humans, with exogenous estrogen is associated with tumors in different organs.[29] Estrogens are generally assumed to function in the tumorigenic process as secondary stimuli or promoters, based on the observation that they stimulate cell growth and the lack of definitive evidence that estrogens or estrogenic chemicals are point mutagens.[30] In spite of the failure to demonstrate conclusively that estrogenic chemicals form covalent adducts to DNA or induce structural DNA mutations, like genotoxic carcinogens, it has been shown that estrogens of diverse structures and biological potencies can function as carcinogens. This evidence includes reports of estrogen-induced neoplastic transformation of cells in culture in the absence of enhanced cell proliferation,[31–33] and the carcinogenic effects of estrogens in the adult hamster kidney,[34] neonatal mouse,[35] and hamster uterus.[36]

Estrogens can induce transient cellular signals in the uterus. Administration of estrogen to an ovariectomized mouse results in organ growth, cell proliferation, and target gene expression in the uterus.[37] When estrogen is withdrawn, uterine size and weight, as well as expression of estrogen-regulated genes, return to approximately the unstimulated state. On the other hand, when estrogens are given to newborn mice, some genes under estrogen control are expressed persistently into adulthood. These genes include lactoferrin, epidermal growth factor, and protooncogenes such as *c-fos*, *c-jun*, and *c-myc*.[37–40] Moreover, perinatal DES exposure can also lead to persistent repression of Hoxa-10 and Hoxa-11, which are responsible for structural abnormalities in the reproductive tracts.[37,41–43] DES and other estrogens, when given early in development (days 1–5), result in a high incidence of epithelial cancers of the uterus in mice at 18–24 months of age.[35] In contrast, mice treated at 20 days after birth show no persistent gene expression change and no increase in cancer incidence in later life. This leads to the question: how does a reversible signal become irreversible in the absence of detectable gene mutation?

As described earlier, during the process of cell differentiation, genes are differentially turned on or off. The methylation or demethylation of a gene's regulatory elements is often a critical determinant of that cell's gene expression pattern. Thus, we have studied the methylation pattern of the promoter of the *lactoferrin* gene in mice treated developmentally at days 1–5 after birth with DES. This treatment results in persistent expression of *lactoferrin* and nearly 100% incidence of epithelial cancers in the uterus of mice at 18 months of age. The pattern of DNA methylation in developmentally treated mice was compared to those treated as adults. Five CpG sites available for methylation occur in a region upstream from the estrogen response element (ERE) in the mouse *lactoferrin* promoter. In the developmentally estrogenized mouse, two sites remain unmethylated; while in the corresponding control (untreated), only one CpG site remains unmethylated.[44] Adult mice treated with the same dose of DES for the same time did not have a change in the DNA methylation pattern of the lactoferrin gene. This is consistent with the inability of such treatment in the adult to persistently change expression of the gene. This methylation alteration pattern was observed only in the uterus, not the liver.

DES also potently represses expression of *AbdB Hoxa* genes in the developing reproductive tract in the mouse. Targeted disruption of *Hoxa-10, Hoxa-11*, and *Hoxa-13* results in region-specific developmental defects along the reproductive tract that are similar to those induced by neonatal DES exposure, suggesting that deregulation of Hoxa gene expression constitutes a mechanism underlying DES teratogenicity.[41–43] To examine whether DNA methylation alteration also involves the downregulation of the hox genes, we studied the methylation patterns of *Hoxa-10* and *Hoxa-11* gene promoters. Methylation assays were performed on 8 CpGs in *Hoxa-10* and 19 CpGs in *Hoxa-11* proximal promoters. The results showed that all these CpGs were unmethylated in both control and DES-dosed mice from postnatal day 5 to day 30. Significant methylation around *Hoxa-10* and *Hoxa-11* promoters was observed only in DES-induced uterine carcinoma.[45] This suggests that DES-induced downregulation of *Hoxa-10* or *Hoxa-11* gene expression is not associated with methylation changes in the proximal promoters of these genes. Thus, DNA methylation modification by developmental DES exposure may be a gene-specific phenomenon.

The altered methylation pattern associated with estrogen treatment during differentiation of uterine epithelial cells provides a mechanism for irreversible expression

of a normally reversible signal. This suggests one possible route for the change from epigenetic to genetic alterations in hormonal carcinogenesis. However, generalization of this mechanism to hormonal carcinogenesis requires studies on methylation and expression of different genes that are important to cell differentiation or proliferation. It has been shown that breast cancer cells have silenced genes that function as tumor suppressor genes and that the silencing is a function of altered methylation.[46] It is not known whether estrogens play any role in the methylation or demethylation of these genes. In fact, very little is known regarding the role of estrogens or other hormones in gene methylation. Yet, estrogen plays a key role in human pathophysiology, and a growing body of evidence suggests that environmental estrogens interfere with several aspects of mammalian (including human) life.[47,48] Therefore, the nature of any impact that estrogen has on gene methylation is of great interest. Recently, Xie *et al.* have provided data on an indirect relationship between the two.[49] They reported that estrogen inhibited catechol-*O*-methyltransferase (COMT) gene transcription. COMT is a ubiquitous enzyme catalyzing the transfer of the methyl group from the coenzyme S-adenosyl-L-methionine (SAM) to one of the hydroxyl groups of catechols in the presence of $Mg2^{+}$. Inhibition of this enzyme results in inhibition of the methylation process.

OTHER ENVIRONMENTAL EFFECTS ON DNA METHYLATION DURING EARLY DEVELOPMENT

As mentioned previously, phytoestrogens, which are naturally occurring estrogenic chemicals, are consumed at high levels in individuals with a plant-based diet. In addition, several phytochemcals such as genistein, an isoflavone found chiefly in soybeans, is consumed in supraphysiologic levels as nutritional supplements and pharmaceuticals. The carcinogenic and anticarcinogenic potential of genistein have been investigated. Genistein intake in adult humans or in rodent models of cancer has been associated with decreased risk of several cancers.[50] However, perinatal exposure has been associated with increased cancer risk. Outbred female CD-1 mice were treated on days 1–5 with equivalent estrogenic doses of DES (0.001 mg/kg/day) or genistein (50 mg/kg/day). At 18 months, the incidence of uterine adenocarcinoma was 35% for genistein and 31% for DES.[51] These data suggest that genistein is carcinogenic if exposure occurs during critical periods of differentiation. It is unclear if the underlying mechanisms for uterine carcinogenesis caused by DES and genistein are the same. However, neonatal exposure in rats to the phytoestrogens coumestrol and equol led to specific gene hypermethylation in the *C-Ha-ras* oncogene in pancreatic DNA, suggesting that this class of compounds can modulate methylation.[52]

Like perinatal exposure to toxic chemicals, drugs, or pharmacological doses of phytochemicals that cause adverse effects in adulthood, other environmental factors such as stress and nutrition early in life can also influence disease risk in later life. For example, perinatal nutritional deficiencies have been associated with coronary heart disease, stroke, hypertension, and type II diabetes, possibly through methylation-related processes modulating glucose and lipid metabolism.[53–55] *In vitro* fertilization (IVF) and cloning techniques in humans and animals change the intrauterine environment during early embryo development, which can result in birth weight alteration, organ abnormalities, and even fetal death.[56–59] Although little is

known about whether these procedures result in epigenetic changes in gene expression, some evidence for this hypothesis exists. For example, selenium deficiency leads to increased *DNMT* activity in carcinogen-treated rat colons and in human colon cancer cells,[60,61] while increased selenium concentrations *in vitro* inhibit *DNMT1* activity.[60] Khosla *et al.* recently reported that tissue culture of preimplantation mouse embryos decreased expression of the imprinted genes *H19* and *IGF-2*. This was associated with a gain of DNA methylation at an imprinting control region upstream of H19.[56,57] In addition, maternal protein deficiency causes DNA hypermethylation in the liver of rat fetuses. Diets lacking the amino acids required for methionine metabolism lead to changes in the methylation status of the fetus.[62]

CONCLUSION

We have described in this review the lessons learned from a series of studies on the effects of perinatal DES exposure and other environmental perturbations early in life, such as nutritional deficiencies and stresses, on methylation patterns and subsequent disease risk in adulthood. Taken together, these findings suggest a mechanism whereby exposure to chemicals or other environmental agents during critical periods of development may induce epigenetic changes in the genome. The critical period may vary depending on the type of environmental exposure and the organs/tissues involved. Aberrant DNA methylation may result in changes in the transcription of key genes and/or may increase the susceptibility of the exposed individuals to a secondary environmental exposure. In either case, DNA methylation status appears to be an early event in the tumorigenic process and may provide a biomarker of increased cancer susceptibility in response to early xenobiotic exposures, diet, or stresses.

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