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Neonatal Exposure to Diethylstilbestrol Alters the Expression of DNA Methyltransferases and Methylation of Genomic DNA in the Epididymis of Mice

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Abstract. Fetal and neonatal exposure to diethylstilbestrol (DES) is known to cause many abnormalities, such as cancer, in the male and female reproductive tracts later in life, and epigenetic mechanisms, such as DNA methylation, may be involved in these processes. In the present study, newborn C57BL/6 male mice were exposed to 3 μg of DES from postnatal days 1 to 5. Subsequently, the expression levels of the DNA methyltransferases Dnmt1, Dnmt3a and Dnmt3b and the transcription factors Sp1 and Sp3, which have been reported to regulate the expression of Dnmts, were examined at days 5, 14 and 30. Furthermore, restriction landmark genomic scanning (RLGS), which can analyze genome-wide DNA methylation, was performed to clarify whether or not aberrant DNA methylation was present in the epididymis of the DES-treated mice at day 30. Increased expression of Dnmt3b was observed at days 5 and 14, followed by increased expression of Dnmt1 and Dnmt3a at day 30, as evaluated by real-time RT-PCR. The expression of Sp1 was also increased at day 30. The RLGS analysis revealed that 7 loci of the genomic DNA were demethylated and 1 locus was methylated in the epididymis of the DES-treated mice. Four of these loci specifically demethylated in DES-treated mice were cloned, and all were found to be located within CpG islands near genes. In conclusion, our results indicated the possibility that DES-induced abnormalities of reproductive organs are associated with altered expression levels of DNA methyltransferases and DNA methylation.

Key words: DNA methylation, Epididymis, Diethylstilbestrol, DNA methyltransferase, Restriction landmark genomic scanning (RLGS)

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THE synthetic estrogen diethylstilbestrol (DES) was prescribed to pregnant women to prevent miscarriage from the 1930s through to the 1970s. However, in 1971, a correlation between DES exposure of mothers and the occurrence of vaginal clear cell carcinoma in their daughters was reported [1]. Subsequently, many other problems of reproductive organs, such as malformations and dysfunctions, were reported to be associated with exposure to DES [2]. Studies using murine models revealed that DES induces similar reproductive abnormalities in mice [3–5], and DES is now widely used in a model for estrogenic endocrine disruptors. Our previous studies revealed that mice treated neonatally with DES or other endocrine disruptors show increased or decreased expression levels of various genes in the epididymis or testes, even in adulthood [6–9]. Since these changes in gene expression last for a long

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time and DES has been reported to be non-genotoxic [10], epigenetic mechanisms may be involved in the changes. In the uteri of mice treated neonatally with DES, altered DNA methylation at CpG sites in the lactoferrin gene promoter [11] as well as persistent elevation of *c-fos* expression and hypomethylation of its exon 4 [12] have been reported. In tumor cells, aberrant DNA methylation occurs frequently and, at that time, increased expression of DNA methyltransferases is often observed [13, 14]. DES-induced alterations in DNA methylation may be associated with the expression of DNA methyltransferases (Dnmts). To evaluate the epigenetic effects of DES, more information about the expression of Dnmts and the changes in DNA methylation, including genome-wide analyses, is required.

To date, several methods for analyzing genome-wide DNA methylation have been developed [15]. Among these, restriction landmark genomic scanning (RLGS) involves two-dimensional electrophoresis of genomic DNA using a restriction enzyme as a landmark. By using a methylation-sensitive restriction enzyme, such as *Not* I, the methylation status of over 1,000 genomic loci can be analyzed in a single experiment [16].

In the present study, we analyzed the expression of Dnmts, as well as genome-wide DNA methylation, in order to investigate the effects of neonatal DES exposure on DNA methylation in reproductive organs. Real-time RT-PCR analysis revealed that the expression levels of Dnmts were increased following DES treatment. Furthermore, alterations in genomic DNA methylation were observed using the RLGS analysis.

Materials and Methods

Animals

Pregnant C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). Newborn male mice were injected subcutaneously with DES dissolved in 25 μl of sesame oil (3 μg/pup/day) from postnatal days 1 to 5. Untreated and vehicle-treated mice were used as controls. At days 5, 14 and 30, the epididymis was excised and stored at –80°C until analysis. All mice were maintained in accordance with the Chiba University guidelines for animal experimentation.

Real-time RT-PCR

Total RNA was separately extracted from the epididymis of 5 mice using the TRIzol reagent (Invitrogen, San Diego, CA). The total RNAs were reverse-transcribed using a QuantiTect Reverse Transcription Kit (Qiagen KK, Tokyo, Japan) according to the manufacturer's protocol. Real-time RT-PCR was performed in a DNA Engine Opticon (MJ Research, Cambridge, MA) using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen). Gene-specific primers were used to determine the relative expression levels of Dnmt1, Dnmt3a and Dnmt3b. The primers for the Dnmts [17] and the other primers used in this study were as follows: Dnmt1 (X14805): forward primer 5'-CCT AGT TCC GTG GCT ACG AGG AGA A-3', reverse primer 5'-TCT CTC TCC TCT GCA GCC GAC TCA-3'; Dnmt3a (AF068625): forward primer 5'-GCC GAA TTG TGT CTT GGT GGA TGA CA-3', reverse primer 5'-CCT GGT GGA ATG CAC TGC AGA AGG A-3'; Dnmt3b (AF068626): forward primer 5'-TTC AGT GAC CAG TCC TCA GAC ACG AA-3', reverse primer 5'-TCA GAA GGC TGG AGA CCT CCC TCT T-3'; Sp1 (AF022363): forward primer 5'-AGG GAG GCC CAG GTG TAG-3', reverse primer 5'-GGC AAG ACG GGC AAT ACC-3'; Sp3 (BC079874): forward primer 5'-TAC TCG CCT CTG GAA CAC CT-3', reverse primer 5'-GCA GAC CAA GAG GCA CAT TAG-3'; β-actin (X03672): forward primer 5'-AGA GGG AAA TCG TGC GTG AC-3', reverse primer 5'-CAA TAG TGA TGA CCT GGC CGT-3'. In all cases, the reactions were performed in triplicate using the same 5 independent samples of epididymal RNA. The real-time RT-PCR results were normalized by their corresponding β-actin content. Data are presented as the mean ± S.E.M.

Preparation of genomic DNA

Genomic DNA was extracted from control or DES-treated mice as described previously [18]. Briefly, the tissues were suspended in lysis buffer (150 mM EDTA, 10 mM Tris-HCl, pH 8.0, and 1% SDS) containing 10 mg/ml proteinase K (Merck, Darmstadt, Germany), incubated at 55°C for 20 min, and then extracted twice with phenol/chloroform/isoamyl alcohol (50 : 49 : 1). Finally, the genomic DNA was precipitated in ethanol, centrifuged into a pellet and redissolved in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.6).

RLGS

RLGS was performed as described previously [18–20]. Briefly, 3 μg of genomic DNA was treated with 10 U of Klenow fragment (Takara Bio, Shiga, Japan) in the presence of 0.4 μM dGTPαS, 0.2 μM dCTPαS (Amersham Biosciences, Buckinghamshire, UK), 0.4 μM ddATP and 0.4 μM ddTTP (Takara). Next, the DNA was digested with 20 U of *Not* I as a methylation-sensitive landmark enzyme (Nippon Gene, Toyama, Japan) and the cohesive ends were labeled with 1.3 U of Sequenase Ver. 2.0 (USB, Cleveland, OH) in the presence of 0.33 μM [α-^{32}P]dCTP and 0.33 μM [α-^{32}P]dGTP (Amersham Biosciences). The labeled DNA was then digested with 20 U of *Pvu* II (Nippon Gene) and subjected to the first-dimension electrophoresis in a 0.9% agarose gel at 230 V for 23 h. The DNA fragments in the gel were treated with 1000 U of *Pst* I (Nippon Gene), and then subjected to the second-dimension electrophoresis at 160 V for 20 h. The gel was then dried and exposed to X-ray film (Kodak XAR 5; Eastman Kodak, Rochester, NY) for 2–4 weeks at –80°C. All experiments were repeated at least twice.

Spot cloning

Four spots in the epididymis of the DES-treated mice were cloned using a modified *Not* I trapper method [21]. In this study, Easy Anchor *Not* I (Nippon Gene) was used instead of the *Not* I trapper. Five parallel 100 μg samples of genomic DNA from the epididymis were digested with 150 U of *Not* I and 120 U of *Pvu* II, and the DNA fragments containing *Not* I ends were collected using Easy Anchor *Not* I. One-fifth of the purified DNA was labeled at *Not* I sites using 1.3 U of Sequenase Ver. 2.0 in the presence of 10 μCi each of [α-^{32}P]dGTP (3,000 Ci/mmol) and [α-^{32}P]dCTP (6,000 Ci/mmol). The labeled portion was mixed with the remaining four-fifths and subjected to the above-described RLGS separation. After exposure to X-ray film for 1 week, the gel was punched out at the targeted spots, and the DNA fragment in each punched-out gel spot was electroeluted. The eluted DNA was ligated into the *Not* I and *Pst* I sites of pBluescript II (Stratagene, CA), and the inserted fragments were amplified by PCR using the M4-RV primer under the conditions of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min for 30 cycles. The PCR products were cloned into the pGEM-T easy vector (Promega, Madison, WI) and the sequences were determined.

Statistical analysis

Data are expressed as the mean ± S.E.M. Statistical analyses were performed using Student's *t*-test. The criterion of significance was set as $p<0.05$.

Results

Real-time RT-PCR

The expression levels of the maintenance DNA methyltransferase Dnmt1 and the *de novo* DNA methyltransferases Dnmt3a and Dnmt3b were examined at days 5, 14 and 30. At days 5 and 14, the expression of Dnmt3b was significantly increased following DES treatment, whereas the expression levels of Dnmt1 and Dnmt3a remained unaltered (Fig. 1). At day 30, the expression levels of Dnmt1 and Dnmt3a were increased, while conversely, the level of Dnmt3b was not increased (Fig. 1). Next, the expression levels of the transcription factors Sp1 and Sp3, which have been reported to stimulate the transcription of DNA methyltransferases, were examined. At day 30, the expression of Sp1 was increased in the DES-treated mice (Fig. 2), and this increase was consistent with the increased levels of Dnmt1 and Dnmt3a. In contrast, the expression of Sp1 was not affected at days 5 and 14 (Fig. 2). The expression of Sp3 was not significantly altered by DES treatment (data not shown).

RLGS analysis

We performed RLGS to analyze the methylation status of the epididymis at day 30 with or without DES treatment. In this analysis, the presence of spots indicates the demethylation of landmark sites and *vice versa*. A total of 1099 spots were compared to identify differences between the control and DES-treated mice. In the epididymis, 7 spots (0.64% of all the spots analyzed) were only detected in the DES-treated mice and 1 spot (0.09%) was specifically detected in the control mice (Fig. 3). The remaining 1091 spots (99.27%) were detected both in the control and DES-treated mice.

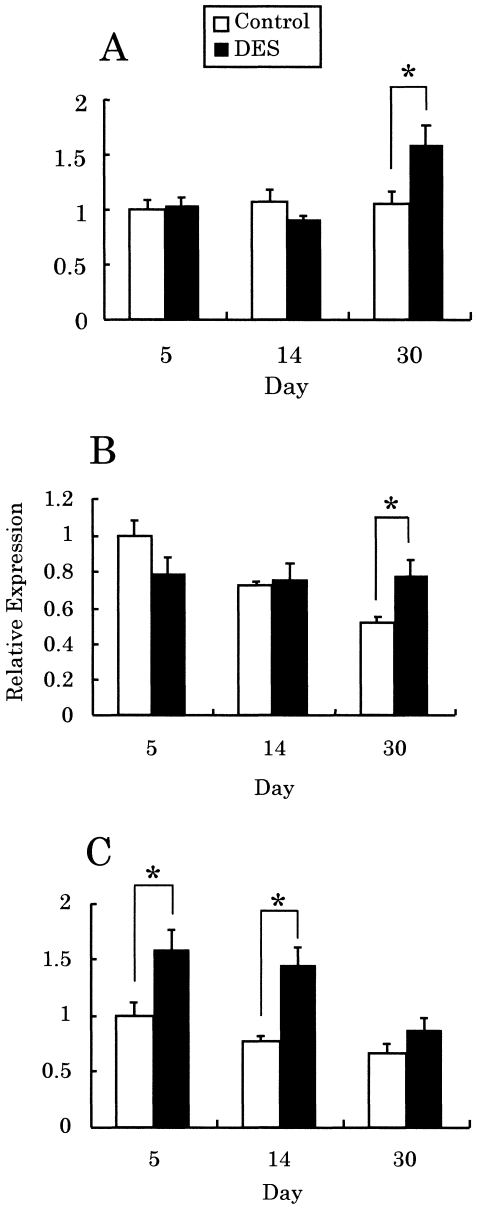

Fig. 1. Effects of diethylstilbestrol (DES) on the mRNA expression levels of DNA methyltransferases.
Newborn mice were exposed to DES from postnatal days 1 to 5, and the expression levels of Dnmt1 (A), Dnmt3a (B) and Dnmt3b (C) in the epididymis were examined by real-time RT-PCR. Data are expressed as the mean ± S.E.M. of 5 epididymides. * $p<0.05$.

Spot cloning

We performed spot cloning using Easy anchor *Not* I and successfully cloned 4 spots. The characteristics of the cloned DNA fragments, such as their genomic loci, GC contents and CpG frequencies [22], were determined and genes near the cloned fragments were identified using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) (Table 1). The 4 cloned spots all had high GC contents (more than 50%), high CpG frequencies (more than 0.6) and satisfied the definition of a CpG island [22], and were located in or near genes. Specifically, spot E4 was located about 3.2 kb upstream of the Mobkl2c open reading frame, spot E5 was located on BC023892, the mouse orthologue of the human c6orf37 gene [23], spot E6 was located on gene model 203, a typical zinc finger-like protein, and spot E7 was located on the translation initiation site of Ifih1 [24].

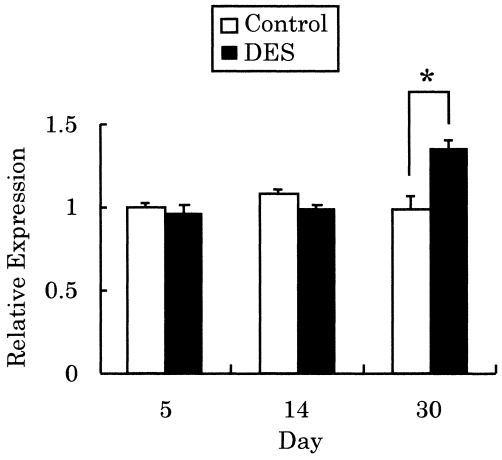

Fig. 2. Effects of diethylstilbestrol (DES) on the mRNA expression level of Sp1.
Newborn mice were exposed to DES from postnatal days 1 to 5, and the expression level of Sp1 in the epididymis was examined by real-time RT-PCR. Data are expressed as the mean ± S.E.M. of 5 epididymides. * $p<0.05$.

Table 1. Characterization of cloned spots in the epididymis

Spot no.	Locus	Gene symbol	Gene name	CpG island
E4	4D1	Mobkl2c	Mps One Binder kinase activator-like 2C	Yes
E5	9E3.1	BC023892	cDNA sequence BC023892	Yes
E6	1G3	Gm203	Gene model 203	Yes
E7	2C3	Ifih1	Interferon induced with helicase C domain 1	Yes

Discussion

In the present study, we examined the effects of neo-

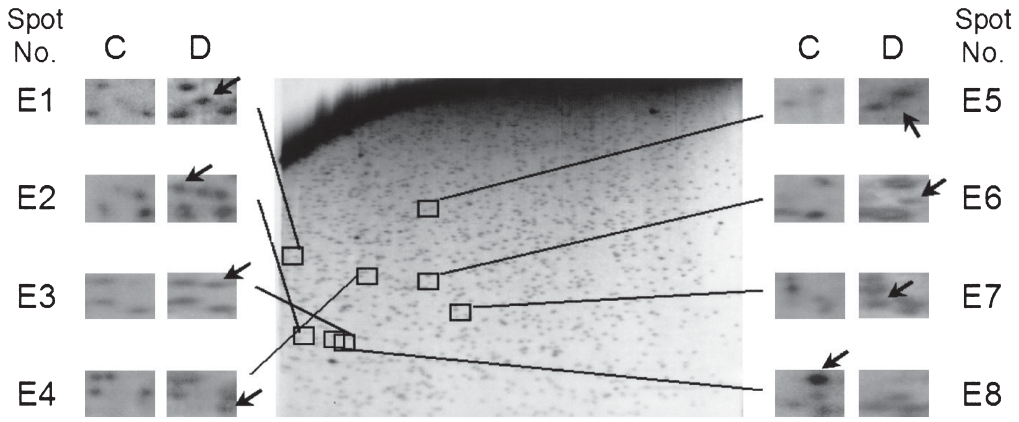

Fig. 3. Restriction landmark genomic scanning (RLGS) profiles of the mouse epididymis with and without DES treatment. Epididymal genomic DNA samples from control and DES-treated mice were subjected to RLGS analysis. Each genomic DNA sample used was a mixture of samples from 3 mice and each experiment was repeated twice. The left and right panels show spots only detected in the control (C) or DES-treated (D) mice. Arrows indicate the differently detected spots.

natal DES exposure on the expression levels of Dnmts and global DNA methylation. Our results indicate that DES exposure during critical stages of development and differentiation causes altered expression of Dnmts and aberrant DNA methylation even in the pubertal stage.

It is important to solve why the expression levels of Dnmts are altered by DES treatment. The transcription of Dnmt1, Dnmt3a and Dnmt3b was reported to be stimulated by the transcription factors Sp1 and Sp3 [25, 26]. In this study, the expression of Sp1 was increased in the DES-treated mice, consistent with the increased expression levels of Dnmt1 and Dnmt3a. In other studies, 17β-estradiol and DES were reported to increase the expression of estrogen-related receptor (ERR) α1 [27] while ERRα1 increases the expression of Sp1 [28]. It is thought that DES alters the expression of Dnmts through Sp1. However, there was no correlation between the expression levels of Sp1 and Dnmt3b. Therefore, other factors besides Sp proteins must be involved. In a previous report indicating that low-dose X-irradiation alters the expression of Dnmts and DNA methylation, ovariectomy and estrogen supplementation had significant effects on the results [29]. More studies are needed to clarify the relationships between estrogenic compounds and Dnmt expression.

In the current study, we performed RLGS using *Not* I as a methylation-sensitive landmark enzyme, since more than 90% of *Not* I sites are located in CpG islands [30]. Previous RLGS analyses using *Not* I have revealed differences in DNA methylation between various types of cells, including Dnmt-deficient embryonic stem cells [31–33]. In the present study, the 4 genes identified in the epididymis were neither genes known to be associated with DES-induced reproductive organ abnormalities nor typical estrogen-responsive genes. It is important to elucidate which factors determine the loci where DNA methylation is affected. In addition to estrogen-responsive genes, such as lactoferrin or *c-fos*, which have an estrogen-responsive element (ERE), genes that have an indirect but close relationship to estrogen may be affected. However, the site preference of Dnmts may be more important than the association of estrogens, and further studies are required to elucidate this problem.

In this study, the correlations between aberrant DNA methylation and reproductive organ abnormalities remained unclear. Recently, however, adverse effects induced by chemicals and concurrent changes in DNA methylation have been reported. For example, exposure of mouse preimplantation embryos to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), which binds to the aryl hydrocarbon receptor, reduced the fetal body weight and increased the methylation levels of the imprinted genes *H19* and *Igf2* as well as the DNA methyltransferase activity [34]. Another report indicated that exposure of gestating female rats during the period of gonadal sex determination to vinclozolin (an antiandrogenic compound) or methoxychlor (an estrogenic compound) induced transgenerational adverse effects on male fertility and altered the DNA methylation patterns in the germline [35]. DES has also

been reported to cause transgenerational adverse effects, such as carcinogenesis [36–38]. In these cases, changes in DNA methylation may also be involved in the adverse processes that occur.

In conclusion, our present results support the recent proposal that, when evaluating the toxicities of endocrine disruptors and other chemicals, epigenetic effects, such as DNA methylation, should be taken into account [39, 40]. We have further suggested that alterations in the expression levels of DNA methyltransferases may be involved in the formation of aberrant DNA methylation.

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