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Genome-wide analysis of epigenomic alterations in fetal mouse forebrain after exposure to low doses of bisphenol A

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ABSTRACT

Bisphenol A (BPA) is one of endocrine disrupting chemicals, being distributed widely in the environment. We have been studying the low dose effects of BPA on murine forebrain development. Here, we have investigated the genome-wide effect of maternal exposure to BPA on the epigenome in mouse forebrain at E12.5 and at E14.5. We scanned CpG methylation status in 2500 NotI loci, representing 48 (de)methylated unique loci. Methylation status in most of them was primarily developmental stage-dependent. Each of almost all cloned NotI loci was located in a CpG island (CGI) adjacent to 5′ end of the transcriptional unit. The mRNA expression of two functionally related genes changed with development as well as the exposure to BPA. In both genes, changes at the transcriptional level correlated well with the changes in NotI methylation status. Taken together, epigenetic alterations in promoter-associated CGIs after exposure to BPA may underlie some effects on brain development.

Endocrine disrupters are defined as chemicals that can interfere with intrinsic hormonal actions in animals. Bisphenol A (BPA), one of such chemicals, is used mainly in the production of polycarbonate plastics and epoxy resins. BPA was detected in the serum of pregnant women as well as fetuses. Many lines of evidence accumulated that BPA causes adverse effects on the reproduction and the development of animals [1,2].

The prenatal exposure to BPA at low doses has been demonstrated to affect brain development in mice, including the development of dopaminergic system [3]. We reported that prenatal and lactational exposures to BPA (3 μg/g powder food) caused a significant decrease of tyrosine hydroxylase (TH)-positive neurons in substantia nigra only in female mice [4]. In addition, we have demonstrated that prenatal exposure to low doses of BPA (20 μg/kg daily, starting from E0.5) perturbed neocortical histogenesis and thalamocortical pathway formation [5,6]: BPA-exposures accelerated neuronal differentiation/migration, being accompanied with the changed expression of several genes involved in proliferation and differentiation of neural progenitor cells. Such effects seen in embryos resulted in abnormal allocation of neocortical neurons and aberrant thalamocortical connections, the latter persisted even after maturation. These observations indicate that BPA affects murine brain development even at low doses. However, what molecular mechanisms underlie still remains elusive.

During vertebrate development it has been postulated that dynamic changes in DNA methylation status are able to regulate tissue- and cell type-specific gene expression [7,8]. Methylation of the cytosine residue at the CpG dinucleotide sites is one of the targets of epigenetic modifications. It is reported that different patterns in DNA methylation are observed at different developmental stages as well as in different types of cells and tissues [9]. Hypermethylation in promoter-associated CpGs often suppresses gene expression via chromatin remodeling [9]. Over the last decade the role of epigenetic status has intensively been studied in developing mammalian cortex. Discovery of mutation of the gene, methyl-CpG binding protein 2 (*MECP2*), in Rett syndrome [10] led us to recognize that the epigenetic changes play critical roles during mammalian brain development. We previously reported how the genome-wide methylation status of developing murine telencephalons changed with time, using restriction landmark genomic scanning (RLGS) method which enabled to demonstrate methylation patterns of NotI loci [11]. Most of NotI sites in the genome should correspond to gene loci, especially CGIs. In 1.7% of these sites, either methylation or demethylation occurred at different developmental stages of the brain and some of them were shown to be tissue-specific. An important role of the epigenetic states has recently been demonstrated in neural progenitor cells during neurogenesis [12]. These findings suggest that methylation in promoter-associated CpGs is regulated by an intrinsic program during brain development.

Two studies revealed that exposures to BPA cause epigenetic dysregulation. In human prostate cancer cell lines, BPA elicited

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hypomethylation in the promoter region of a gene, resulting in changed expression of the gene [13]. In variable yellow agouti (A^{vy}) mouse, maternal exposure to BPA decreased the methylation level at 5′ long terminal repeat (LTR) of an intercisternal A-particle elements (IAP) retrotransposon that is inserted within *agouti* gene [14]. Consequently, such demethylation changed the ectopic promoter activity of the LTR, followed by perturbed expression of a flanking gene. These findings led us to hypothesize that prenatal exposure to BPA may affect the epigenome in developing mouse forebrain as well and may change the transcription level of the genes. In order to address the issue we have attempted to study genome-wide alterations by RLGS.

Materials and methods

Animals and treatments. ICR/Jcl mice were housed in a temperature-controlled (24 °C) animal facility, with a 12:12-hr light:dark cycle. All animal studies were approved by the Ethics Committee for Animal Experiments at Kyoto Prefectural University of Medicine, and the animals were handled according to the institutional guidelines and regulations. Adult females were mated with normal males, and the morning when a vaginal plug was observed was designated embryonic day 0 (E0). Pregnant mice were randomly divided into two groups. For the BPA-exposed group, pregnant mice ($n = 3$) were given 20 μg/kg of body weight BPA (Wako, Japan) dissolved in sesame oil with subcutaneous injection once daily from E0. The control group ($n = 3$) received a subcutaneous injection of the same amount of sesame oil as that used in the BPA-exposed group. The dams were sacrificed at E12.5 or E14.5 under deep anesthesia with sodium pentobarbital. Dissected embryonic telencephalons were snap-frozen in liquid nitrogen, and stored at −80 °C until use. The tissues of either 15 or 6 offspring from three pregnant mice in each group were mixed, respectively. The former was used for genomic DNA preparation, and the latter for RNA preparation.

RLGS and cloning of spot DNA. Details of the RLGS method and the spot DNA cloning are described in [15]. Briefly, 3–5 μg of the genomic DNA extracted from the frozen tissue was first digested with NotI (TOYOBO, Japan), followed by radio-end labeling. The DNA was then digested with EcoRV (Nippon gene, Japan), and subjected to first dimensional electrophoresis in a 0.8% agarose disc gel. DNA fragments in the disc gel were treated with HinfI (TaKaRa Bio, Japan). The second dimensional electrophoresis was carried out in a 6% non-denatured polyacrylamide gel. The dried gel was exposed to X-ray film. Cloning and identification of spot DNAs were carried out as described in Supplementary material 2.

Methylation-sensitive quantitative PCR (qPCR). Methylation-sensitive qPCR was carried out as described in Supplementary material 2.

Preparation of RNA and reverse-transcription mediated qPCR. Total RNA from fetal mouse forebrain was extracted and purified with RNeasy Microprep kit (Qiagen, USA) according to the manufacturer's instruction. First-strand cDNAs from 1 μg of RNAs were prepared using iScript cDNA Synthesis Kit (BioRad Laboratories, UK). Quantitative PCR was performed with the synthesized cDNAs, SYBR Premix Ex Taq (TaKaRa Bio), PRIZM7000 (Applied Biosystems), and the primer sets described in Supplementary material 1. The experiment was repeated three times.

Results

RLGS

The genomic DNAs were prepared from the forebrain of BPA-exposed mice and vehicle control mice, at two developmental stages (E12.5 and E14.5). RLGS procedures were carried out with the landmark restriction enzyme NotI. The representative RLGS profile is shown in Fig. 1A. If CpG methylation of NotI sites in the genome occurs with time of development or by the exposure to BPA, the corresponding spots on the profile disappear because NotI cannot digest methylated DNA. NotI, therefore, was used as the landmark of CpG methylation in the genome. We surveyed methylation status of 2500 NotI loci (spots) that were distinguishable from each other.

Signal intensity of total 48 spots (1.9%) changed between the control group and the BPA-exposed group. Representative photos are shown in Fig. 1B. There were various patterns in terms of changes in spot intensities, and we were able to classify them into eight categories as summarized in Table 1. Out of the 48 spots, 22 spots (0.9%) and 18 spots (0.7%) were induced specifically by BPA-exposures, at E12.5 and at E14.5, respectively. In addition, eight spots (0.3%) changed at both stages. In each category, a variety of spot behaviors was observed as shown in Fig. 2. The increase of spot intensity in the BPA-exposed group indicated that the exposure caused DNA demethylation at primarily hypermethylated loci. On the other hand, the decrease showed the induction of DNA methylation at the loci. In more than half of changed spots the

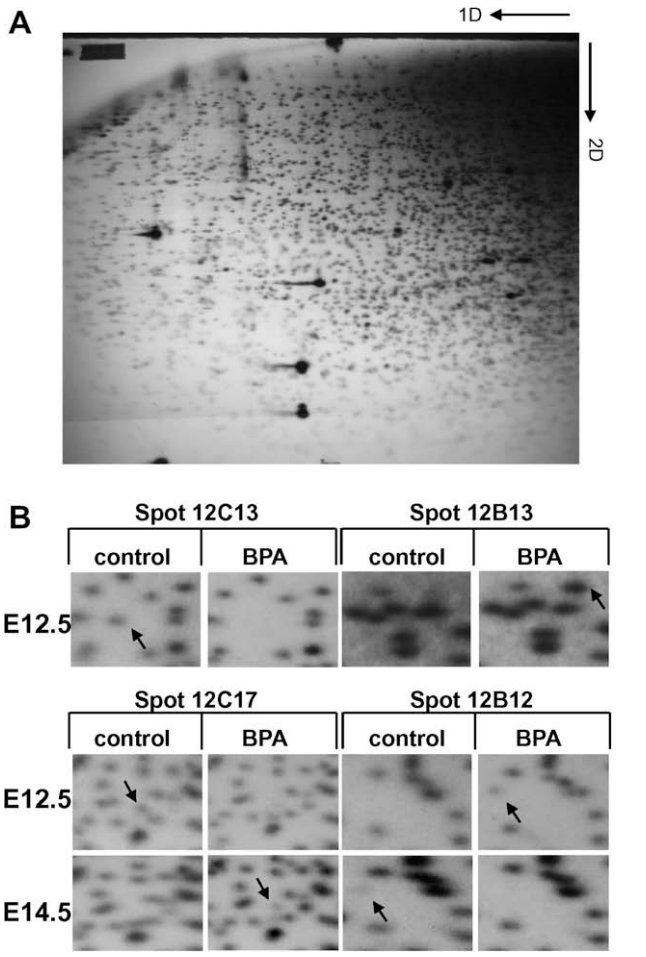

Fig. 1. A representative profile of RLGS and the changing pattern of spots in BPA-exposed mouse telencephalons. (A) RLGS pattern of mouse telencephalons at E12.5 in the control group. 1D and 2D show directions of the first-and the subsequent electrophoresis, respectively. (B) Examples showing changes of the spot patterns in BPA-exposed mouse telencephalon. Two spots, 12C13 and 12B13, (arrows) changed only at E12.5. Two spots, 12C17 and 12B12 (arrows) showed oppositely-oriented behaviors during development (E12.5 and E14.5) between the control group and the BPA-exposed one.