EXPOSURE OF PREIMPLANTATION EMBRYOS TO 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN (TCDD) AFFECTED FETAL DEVELOPMENT

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Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a ubiquitous environmental contaminant that induces a wide spectrum of toxic responses including severe weight loss, fetotoxicity and teratogenicity. TCDD may affect fetal body weight by dosing to mother[1], and seems to have direct effects on preimplantation embryos[2]. However, it is not yet clear whether such an exposure of preimplantation embryos to TCDD affects postimplantation development and growth of fetuses or pups.

During preimplantation stage, extensive changes of methylation take place in the genome but do not occur in so-called imprinted genes[3]. Imprinted genes, such as insulin-like growth factor 2 (Igf2), H19, or growth factor receptor binding 10 (Grb10), may be particularly susceptible to changes of environmental condition that occur during preimplantation development[3, 4]. Igf2 encodes a fetal growth factor and H19 is believed to play a role during embryogenesis and to share similar regulatory element with Igf2 possibly by an enhancer competition system[4, 5]. The product of Grb10 affects the IGF (Insulin-like growth factor)/INS (insulin) axis[6]. Aberrations of Igf2, H19 and Grb10 expression were reported to be associated with fetal development, and perturbation of the methylation process of Igf2, H19 and Grb10 genes during preimplantation stage could result in deregulation of development of embryos at postimplantation stages[3, 4].

In the present study, we exposed the preimplantation embryos to TCDD and transplanted them to recipients to investigate the effect of TCDD in the postimplantation period. We also examined the expression of the imprinted genes, Igf2, H19, and Grb10, and detected the methylation status of a methylation mark in H19 gene.

Materials and Methods

Embryo collection, TCDD treatment and transplantation

Animal experiments were performed according to the guidelines for animal welfare at the National Institute for Environmental Studies. Male and female ICR mice, 9 to 10 weeks old, were purchased from Charles River Inc. (Tokyo, Japan). The animals were provided access to food and water *ad libitum* and kept on a 12 light:12 darkness cycle. Female mice were superovulated with 5 IU PMSG, and given 5 IU hCG 48 h later. The superovulated females were mated with males.

Embryos at the 1-cell and 8-cell stage were collected from the oviduct at approximately 18 h and 66 h, respectively, after hCG administration. TCDD dissolved in DMSO was added to M16 medium (sigma), and embryos were cultured to blastocyst stage in M16 medium containing 10 nM TCDD covered with mineral oil in a humidified atmosphere of 5 % CO_2 and 95 % air at 37 °C. After incubating the blastocyst embryos with clearly visible blastocoel cavities that had developed from 1-cell and 8-cell stage,

respectively, were transferred into uteri of mature recipient ICR females. To avoid artifacts due to fluctuations in the maternal contribution, the control (7 blastocysts /horn) and TCDD exposed (7 blastocyst/horn) blastocysts that were compared were transferred contralaterally to recipient females.

Semiquantitative RT-PCR

Semiquantitative reverse transcription-polymarase chain reaction (RT-PCR) analysis of the mouse embryos was carried out in the following manner[7]. Total RNA was purified from whole fetus that dissected at embryonic day 14 (E14) with Trizol (Life Technologies, Rockville, MD), reverse-transcribed with Superscript II (Life Technologies, Rockville, MD) according to a supplier's protocol. The primer sets used in this study are described as follows: Grb10, forward, cctgattgctggaaagaagc; reverse, cacgagaccttgttgcttga; H19, forward, gcactaagtcgattgcactgg; reverse, ctgcttccagactaggcgag; Igf2, forward, gtccagcaaccatcagtgaa; reverse, ggcactgaagcaatgacatg; G3PDH, forward, agttggagattgttgccatcaacgac; reverse, gggagttgctgttgaagtcgc. The reaction program for Grb10, H19, Igf2, and G3PDH consisted of 22 cycles (15 cycles for H19, 26 cycles for Igf2) of 94 °C for 60s, 55 °C for 60s, and 72 °C for 60s. Under the above conditions, the PCR reaction was conducted in the exponential range of amplification for each set of primers. The density of the PCR products of Grb10, H19, and Igf2 was normalized to that of G3PDH, and the ratios were used for comparison.

Reactions performed with template but without reverse transcription and reactions in the absence of template were utilized as negative controls.

A quantitative HhaI-PCR assay to detect methylation of H19 gene

Methylation status of a methylation mark in H19 gene was detected by the essential the same method as previously described[8]. Briefly, after isolation of DNA, samples were digested with NotI alone (to reduce the size of the DNA) or NotI plus the methylation-sensitive enzyme HhaI. The HhaI site of H19 fragment was made of the primers: forward, tatgcctcagtggtcgatatg; reverse, gaatgctatgcctgagtgacc. A control fragment that does not contain the HhaI site was made with the alternate forward primer, tatcgtggcccaaatgct, and the same reverse primer. The reaction program was as follows: 94 °C/5 min x 1 cycle; 94 °C/1 min, 50 °C/2 min, 72 °C/3 min, x 5 cycle; 94 °C/0.5 min, 50 °C/ 2 min, 72 °C/1.5 min, x 25 cycle; 72 °C/6 min, x 1 cycle. The PCR products were separated by electrophoresis to detect the amplified products.

Results and Discussion

The effects of TCDD on E14 fetus by exposure at preimplantation stage were evaluated on the basis of viability and weight. No significant difference in viability at blastocyst stage and at E14 fetus was found between control embryos and TCDD (10nM)-exposure embryos. In E14 embryos exposed to TCDD from 8-cell to blastocyst stage, fetal weight was similar to that of the controls. However, in E14 embryos exposed to TCDD from 1-cell to blastocyst stage, the fetal weight was significantly 10 % lower than that of the controls.

The relative Igf2, H19, and Grb10 mRNA levels in embryos were then determined at E14 by a semiquantitative RT-PCR technique. E14 embryos exposed to TCDD did not show any significant change in Igf2, H19, and Grb10 mRNA expression from 8-cell to blastocyst stage, but showed a significantly reduced expression for H19 and Grb10 mRNA expression from 1-cell to blastocyst stage. There was a tendency of reduction of Igf2 expression without statistical significance.

It is reported that the allelic methylation status of a differentially methylated region upstream of the H19 gene is essential for the imprinted expression of both H19 and Igf2[4, 5]. The imprinting status of H19 had been reported[9]. To investigate whether methylation level in H19 were associated with the

difference in H19 expression between control embryos and embryos exposed to TCDD from 1-cell to blastocyst stage at E14, we assessed the methylation status of H19 at a methylation-sensitive HhaI site in a 5 kb up-steam region from the transcription-start site. However, no difference in methylation level at this methylation-sensitive HhaI site was found between control embryos and TCDD-exposure embryos.

These results indicated that in preimplantation stage, probably during the 1-cell to 8-cell stage, TCDD appeared to have some effects on the genes, such as H19 and Grb10, which are responsible for postimplantation development. The H19 seems more sensitive to environmental condition than Igf2. Nevertheless, we could not find any association between the reduction of H19 expression in TCDD-exposed embryos at E14 and change of methylation level in H19 gene.

In summary, the present study reveals that exposure of preimplantation embryos to TCDD from 1 cell to blastocyst stage affects the postimplantation development. The fetal weights of TCDD exposed embryos at E14 were lower than that of control embryos. The expressions of H19 and Grb10 were lowed by TCDD exposure. However, change of the methylation status at the methylation-sensitive site of H19 was not detected in this study.

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