In normal development, each gene correctly expresses under temporal and spatial regulation. Teratogenic agents among environmental contaminants induce aberrated gene expression and consequently lead to congenital malformations. Therefore, it is urgent to identify molecular markers for the detection of teratogenic agents’ effects.

We analyzed mouse 39 hox gene expression in teratogenic factor exposed embryos. We found that 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) and retinoic acid (RA) affected differentially expression of hox. Unlike the RA-effects, TCDD led to broad repression of all hox loci. This different effect was also detected in miRNAs (microRNAs) expression in hox loci. Our results indicate that this irregular hox expression is a cause of congenital malformation, and suggest that monitoring of all hox expression works as a marker for environmental contaminants, including teratogenic effects.

Key words: teratogenic agent; gene expression; transcriptional regulation; hox; morphogenesis

We are at serious risk of being exposed to deleterious agents, because the numbers of them in the environment are increasing. If these environmental contaminations are maternally taken in at high levels, congenital malformation will appear frequently in infants and sometimes in their progeny.1) The appropriate temporal and spatial expression of suites of developmental genes is critical to normal development and physiology. Teratogenic agents such as dioxin lead to eliciting irregular gene expression through ligand-induced transcription factors.

Ahr (aryl-hydrocarbon receptor) is a ligand activated transcription factor. Halogenated aromatic hydrocarbons such as TCDD and some tryptophan-derived agents have been identified as AHR-agonists.2) These agonists lead to translocation of AHR from the cytosol to the nuclei and to formation the hetero-dimers with ARNT (AHR nuclear translocator).3) After hetero-dimer formation, they form transcriptional complexes with co-activators or co-repressors partially overlapping nuclear hormone receptors,4) and regulate some target genes expression.5) Ahr is essential for normal development,5,6) but excessive ligand intake leads to teratogenic malformation in across species. Human and mouse embryos exposed to excessive dioxin levels show craniofacial anomalies, endocrine and reproductive toxicity, cardiovascular disease, and multisite cancer.1) These multi-phenotypes suggest that teratogenic factors lead to inadequate action of ligand-activated transcription factors, and accordingly their target genes’ batteries are incorrectly regulated. Still, information on AHR regulated genes is very restricted and the molecular mechanism of malformation is not clearly identified. Recently it was reported that some developmental gene expression is affected in dioxin exposed mouse embryos by microarray analysis.8) This group also screened the dioxin response elements (DREs) using bioinformatics, but it was not clear whether the affected genes had responsive elements, or were regulated directly by AHR.

Hox genes are highly conserved gene family containing the homeo-box. In mammals, 39 hox transcription factors clustered on four chromosomal loci, hoxa through hoxd, are essential in specifying the positional identities of cells.9) The temporal and spatial pattern of hox gene expression is often correlated to their genomic location within each locus, a property termed collinearity.10) This proper hox expression is controlled by several mechanisms: histone modification, several cis-elements, trans-acting factors, and exogenous stimuli such as RA.11–16) Once deviance from this regulation leads to varied phenotypes having similarities in the case of teratogenic agent exposure. In utero TCDD exposed mice shows disruptions of ventral and dorosolateral prostate budding.17) These phenotypes are found in hoxd13 paralog mutants. Mice lacking hoxb13 show abnormalities of the ventral prostate, and hoxb13 and hoxd13 double knockout mice exhibit severe hypoplasia of the duct tips.18) These phenotypic similarities are found in other organs, the craniofacial abnormalities are in hoxa2 knockout mice and hoxa7 or hoxb7 transgenic mice,19–21) and the kidney defects are found in hox11 paralog and hoxd cluster mutants.22,23) These results suggest that dioxin leads to irregular hox expression and induces malformations in some organs, but the relationship between hox expression and dioxin is not yet reported.

We investigated the expresional changes of 39 hox genes in TCDD- and RA-exposed mouse embryos. These teratogenic factors have differentially affected 39 hox and also miRNAs expression in the hox loci. Our results indicate that the monitoring of hox expression performs as a biological maker in short-term teratogenic agent intake, and suggest that a portion of irregular hox expression causes malformation in some organs.

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Materials and Methods

Animals. Pregnant 6 to 7 month-old C57BL/6 mice were orally administered all-trans retinoic acid (Sigma, MO, USA) and TCDD (Supelco, PA, USA) at a dose of 40 mg/kg or 20 μg/kg body mass dissolved in sesame oil (Sigma) at E10.5 respectively. Control females received DMSO (Sigma) suspended in sesame oil. Six h after administration, animals were sacrificed under the guidelines for the animal care and use committee of the University of Tokyo, and embryos were isolated.

Semi-quantitative RT-PCR. Total RNAs were isolated using TRIzol reagent (Invitrogen, CA, USA) from whole embryos. After DNase I treatment, 1.25 μg total RNAs were reverse-transcribed using SuperSript III (Invitrogen) according to manufacturer’s instructions. The PCR condition was 96 °C for 30 s, 62 °C for 30 s, and 68 °C for 30 s using Titanium Taq DNA polymerase (Clontech, CA, USA). β-actin and rp49 genes were amplified 26 cycles for normalization. Primer sets for semi-quantitative PCR are shown in Supplemental Table 1 (see Riosci. Biotechnol. Biochem. Web site). PCR products were loaded on agarose gels, and images were analyzed using Gel-Pro analyzer (Nippon Roper, Tokyo, Japan).

Statistics. All results are presented as mean ± SEM. All statistical analysis was done using Excel software. Statistical differences were determined by Student’s t test. p < 0.05 was considered significant.

Results

Teratogenic agents showed different effects on the fetus under differing dosages and timing of administration.24,25 We used reported dosages of TCDD and RA having teratogenic efficacy in E10.5 mice.20 Additionally at this stage, all hox genes are expressed.27

At 6 h after oral administration, semi-quantitative RT-PCR using total RNA from three or four E10.75 embryos of identified sex was performed. All agent-stimulated littersmates had beating hearts and the resorption of the embryo was not found, but we detected several phenotypes, such as the craniofacial anomalies and kidney defects at E18.5 (Fig. 1).

The administrated RA had the teratogenic effects for 6–8 h on the developing embryos. This limited effect is caused by P450 enzyme dependent metabolization.28 There are three genes of cyp26 in the mouse genome, annotated cyp26a1, b1, and c1, and they are essential for the metabolism of RA in embryo genesis.29 These gene expression patterns are different, but are up-regulated after RA intake due to RARE-dependent gene activation in each gene.20 In the case of TCDD, other groups of P450 enzymes are up-regulated and function partly in xenobiotic detoxification.31 We found that catalytic gene expression was up-regulated under these dosages and for this stimulation time (Fig. 2A and B). In the case of RA administration, cyp26a1 expression was more activated than cyp26c1. This difference is thought to be a reflection of cis-acting elements, because there are two RAREs in cyp26a1.32 Intake TCDD, cyp1a1 expression was more clearly induced than in the control and RA administrated embryos. This induction was dependent on cooperative several XREs (Xenobiotic responsive elements) and basal promoter activity in cyp1a1.33

Next we analyzed 39 hox expression under this condition, which induced xenobiotic gene expression. The 300–500 bp regions were amplified in our assay, and all products were detected less than 32 cycles. There was no notable difference between male and female under the same treatment except for hoxc9 expression. In hoxa cluster genes, 3′ located hoxa1 expression was most induced by the administration of RA (Fig. 3A). This induction was gradually propagated to 5′ located hoxa9. In contrast to this induction, more 5′ located hoxa11 expression was reduced and most 5′ located hoxa13 did not clearly change, and while RA effects, up-regulated hoxa cluster genes were not detected in the TCDD exposed fetuses. Rather, some hoxa cluster genes, hoxa2, hoxa4, hoxa5, and hoxa9, were down-regulated. However, the degree of repression was not found to be in clear correlation with the alignment of chromosomal location. RA affected hoxb cluster genes in the same manner in the hoxa cluster (Fig. 3B). The 3′ located genes were more sensitively activated than the 5′ located genes. In hoxb cluster genes, hoxb4 expression

Fig. 1. Teratogenic Agents Affected Morphogenesis in E18.5 Mice.

After 8 d from one-shot administration at E10.5, kidneys were recovered from control (A), 40 mg/kg RA (B), and 20 μg/kg TCDD-treated embryos (C). Typical hydronephrosis was found in TCDD-treated embryos. Control mice had tails over the hind legs (D), but all RA-treated mice had short tails (E; arrow), and craniofacial abnormalities were found in some cases (F).
was most positively effected 6 h after administration. This declination to more 5’ location might be a reflection of the exposure period. In contrast, some amount of hoxb cluster gene reductions, except for hoxb4, was found in the TCDD exposed embryos, but these changes showed no significant differences. The effect on hoxc cluster expression is shown in Fig. 4A. Gradually activated expression was detected in hoxc4 to hoxc10 in the RA-treated embryos. Unanticipatedly, one control-treated male embryo had the lowest expression; no difference in hoxc9 was clearly identified. In the TCDD-treated embryos, hoxc cluster expression was not spread, but more restricted. The significant differences were detected in hoxc6 and hoxc12. TCDD exerted an inhibitory influence but these effects had no relation to collinearity in this hoxc locus. These two teratogenic agents extensively affected hoxd cluster expression (Fig. 4B). 3’ located hoxd1~d3 expression was up-regulated by RA in the manner of other cluster genes. In contrast to the hoxa, b, and c cluster, reductions of expression was detected in hoxb8 to hoxb13 palarog in the d cluster in both administrated embryos. Especially in the TCDD-treated embryos, the differences were more remarkable than in the RA-treated embryos.

The two teratogenic agents had broad effects on the expression of the hox cluster genes. In these four chromosomal loci, there are some annotated miRNAs. As for this miRNA effect on hox cluster gene expression, it is unknown whether teratogenic factors affect miRNA expression. We investigated the primary transcripts levels of these miRNAs (pri-miRNAs) (Fig. 5). The miR-196b, located between hoxa9 and hoxa10, was modulated negatively by TCDD. This negative effect of TCDD was detected in pri-miR-196a2, but not in pri-miR-196a1. Another miRNA family that regulates hox expression is miR-10. The miR-10a and miR-10b, located in the hoxb and in hoxd clusters respectively, were up-regulated by RA treatment. The range of difference was clear at pri-miR10a, but not found in TCDD-treated embryos. miR-615 is another member of miRNAs in hox loci, but this primary transcript was not detected in our assay (data not shown). Our results indicate that the two teratogenic agents had differential effects on all four hox loci.

Fig. 2. RA and TCDD Led to Xenobiotic Enzyme Expression. A, Expression of teratogenic agents inducible xenobiotic enzyme genes was detected by semi-quantitative RT-PCR using control- (lane 2–4), 40 mg/kg RA- (lane 5–7), and 20 µg/kg TCDD-treated (lane 8–11) embryos as templates. No reverse transcription step was a negative control for RT-PCR (lane 1). B, Fold changes in expression were relative to the control-treated embryos after β-actin normalization. The cyp1a1 value is omitted due to no amplification in the control and RA-treated embryos. In all panels, two-tail t-test results are indicated by (*) p < 0.05 and (**) p < 0.01 relative to the control-treated embryos.

Fig. 3. RA and TCDD Affected hoxa and hoxb Cluster Gene Expression. A, The upper panel is a representation of the genomic organization of mouse hoxa cluster genes. Each box is a 13 paralogous gene in the hoxa cluster. The left side is a 5’ region of a genomic direction. The lower panel shows expression changes in hoxa cluster genes. B, The upper panel shows the genomic organization, and the lower panel shows expression changes in mouse hoxb cluster genes. The expression values were normalized to the expression levels of β-actin, and are presented as fold change ± SEM (n ≥ 3) relative to that of the control embryos.
Discussion

Our results indicate that the two teratogenic agents induced irregular hox expression that in quite different manners and that this mirrors part of the developmental aberrant process.

First we detected expressional changes in all 39 hox genes in TCDD-treated mouse embryos. TCDD has broadly negative effects in all hox loci, but not in the same manner as excessive RA. In the hoxd cluster, the hoxd12 and hoxd13 expression levels were down-regulated significantly in the TCDD- and RA-treated embryos, but the expression domains had quite different patterns in the limbs, where these hox genes dominantly express (data not shown). This difference was detected in another hox expression domains (data not shown). These results indicate that these teratogenic factors have a negative effect on hox expression, but that the repression mechanism is quite different.

Hox expression is controlled in several ways. First, post-transcriptional modification of histone proteins mediates epigenetic regulation of gene expression.34) Many transcription factor genes, including hox, are located in the bivalent domain of histone H3.11) This characteristic feature is linked to the functions of trithorax or Polycomb, and critically maintains the expression of transcription factors.35) In the case of RA-exposure, some hox expression is regulated by...
cis-elements involving ligand-activated transcription factors.\textsuperscript{15,16} Recently, it was reported that H3 methylated states are regulated by many demethylases and, that these enzymes have important roles in development.\textsuperscript{36} KDM5A is one of the demethylases that have effects on H3K4me3/2.\textsuperscript{37} Since KDM5A interacts with histone methyltransferase and demethylase and of histone states, and whether AHR forms a complex cis directly regulates this hox expression, it has been reported that many dioxin responsive elements (DREs) were annotated in the conserved regions between human, mouse, and rat from bioinformatics.\textsuperscript{39} The reported DREs are located in the hoxa2, hoxb4, and hoxb7 regions and it has been suggested that AHR directly regulates this hox expression, but it is not clear and it is necessary to determine whether these elements function in vivo, whether dioxin affects the modification of histone states, and whether AHR forms a complex with histone methyltransferase and demethylase and directly regulates hox expression through the DREs.

After these chromatin modifications, the platforms the trans-acting factors are made and work as cis-elements. Since these cis-elements binding transcription factors maintains hox expression, cdx is one of the factors that genetically lie upstream of hox.\textsuperscript{40} In our results for short-term administration, we detected that cdx family expression is also affected by two teratogenic factors, but in different ways (data not shown). It remains possible that other transcription factors are involved, but different cdx family expression might be one cause of the different hox codes in the two teratogenic agents’ effects.

MiRNAs play important roles in the expression of hox.\textsuperscript{13} Our results indicate that the primary transcript alterations of miRNAs were different in the two teratogenic agent treatment. RA-exposure, miRNAs expression has a correlation with near hox expressions. However, TCDD also negatively affected miRNA levels, and had no correlation to near hox expression. More analysis of the relationship between these miRNAs and hox is needed, but these different modes of miRNA expression might also lead to the different hox codes in the two teratogenic agents’ effects. Hox and miRNAs regulate target gene expression, other cohorts are up or down-regulated by teratogenic factors. Here we investigated expression changes in the mab21 family that we and other group have identified as the HOX target gene.\textsuperscript{41–43} Although the mab21 family is reported to be one of the developmental genes whose expression is affected by dioxin,\textsuperscript{44} we did not significantly detect these expression changes (data not shown). This difference reflects the treatment time from administration. For these reasons, it is necessary to perform time course experiments and to analyze other target genes expression. As at least 3,000 locations have been reported as the sites occupied only by HOXA13.\textsuperscript{43} Identification of direct target genes should first be attempted, and an understanding of the hox gene network should lead to clarification of the phenomena attributed to teratogenic agents.

Our results indicate that the teratogenic agents affect the expression of transcriptional factors in development under short-term administration. These early effects propagate their target genes expressions. It is necessary to explore whether other agents such as persistent organic pollutants (POPs) have potentiality to affect hox expression, but the hox code should be regarded as a molecular marker to monitor environmental contaminants having teratogenic effects.

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