Elucidation of endocrine disrupting mechanism of dioxin and related compounds for health risk assessment

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Introduction

Inflammable wastes have been subject to combustion by municipal and industrial incinerators in Japan. During the late 1990s the large-scale operation of incinerations caused serious public concerns, particularly in the vicinity of the metropolitan area in which industrial waste incinerators are bristled with. In February 1999, a popular TV news program broadcast the detection of dioxins in green vegetables, which raised public concern about the safety of consuming agricultural products or living close to incinerating facilities. The Japanese government organized cabinet-level board meeting to formulate the fundamental policy on how to cope with dioxin issues. Accordingly, the joint dioxin risk assessment committee under the Ministry of Health, Labour and Welfare (MHLW) and the Ministry of the Environment (MOE) established a tolerable daily intake (TDI) of 4 pg TEQ/kg-bw/day in June 1999[1], after seriously studying the revision of TDI proposed by the WHO Consultation in 1998[2]. The Special Measures Law on Dioxin Control stipulating environmental standards and release limits calculated from this TDI value went into force in January 2001. At the same time, since the law mandates constant monitoring of the environment by the municipal government and compliance to the more strict emission limits for incinerators and furnaces, it will impose heavy responsibility and burden on both the government and private sectors. There is a current reaction against these environmental-cautious actions, represented in a recently-published book, 'Dioxin –the end of a myth', implying that dioxins are not as dangerous as have been thought and that too much budgets have been directed to the researches on risk assessment as well as counter-measures.

The important point on health risk assessment for dioxins as well as environmental endocrine disruptors is that we should scientifically evaluate whether the actual exposure level from food and environment may pose a threat to human health not only for the present but also for the future generations. We formulated a research project, called CREST project with support from the Japan Science and Technology Agency, in order to obtain experimental evidence for risk assessment as well as for the mechanism of toxicity.

We thus investigated the dose-response relationships of certain 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-like compounds for eliciting various endpoints. First, we administered TCDD or coplanar polychlorinated biphenyl (PCB) to rats and mice during the sensitive period from fertilization to birth. Second, we studied what kinds of adverse effects could be observed in terms of reproductive/developmental effects, cognitive/learning abilities and immune functions. Third, we focused on the actual toxicity phenotypes found at the whole body and organ/tissue levels by a forward toxicology approach, and tried to narrow down appropriate phenomena, to study the

mechanism on the molecular basis by the reverse toxicology approach. In this presentation, I will summarize the outcome of the CREST project.

Dose-Effect relationship

In most experimental protocols, we administered TCDD or PCB congeners/isomers, as a single oral dose on GD 12.5 and GD 15 in pregnant mice and rats, respectively, according to the well-established protocol in earlier studies. We examined various endpoints of toxicity in reproduction, brain function and immune function with the estimated body burden of dioxin, according to essentially the same principle adopted by the WHO consultation[2].

It has been reported that female as well as male reproductive organs are one of the most sensitive targets of TCDD. In the present study, shortening of anogenital distance and a decrease in ventral prostate weight were found in pups as early as PND 2 until PND 120, whose dams were administered TCDD at a dose of 50 ng/kg and 200 ng/kg, respectively. On PND 49, androgen receptor mRNA and 5α-reductase II mRNA levels in the ventral prostate were found to be suppressed and elevated, respectively, in a group of pups whose dams were administered a TCDD dose as low as 12.5 ng/kg [3]. This endpoint was adopted as one of the most sensitive marker for the health risk assessment done by the Joint Expert Committee of FAO/WHO in 2001 [4].

Regarding the maturation of female rats, we found that administration of 200 or 800 ng TCDD/kg, the dose was confirmed not to exert malformations, facilitate ovary weight gain, vagina opening and the age at first estrus, in a dose-dependent manner. First estrus was 7-12 days earlier in the 800 ng TCDD/kg-exposed offspring than the vehicle-exposed controls. In addition, corpus luteum formed after ovulation were found approximately 10 days earlier in TCDD exposed female offspring. We next examined ovarian compensatory hypertrophy (OCH) as an indicator of the maturation of the LH/GnRH-generating system in the pituitary and hypothalamus. Exposure to TCDD accelerated the onset of OCH in female offspring in a dose-dependent manner. In the 800 ng TCDD/kg-exposed animals, hypertrophy, characterized by hyperovulation and a drastic increase in ovarian weight after hemi-ovariectomy, was observed at PND 27 to 30, which was 10 days earlier than that in vehicle-exposed animals ($p<0.001$). These results indicate that perinatal exposure to low doses of TCDD caused precocious puberty in female rats, including maturation of not only the gonads and genitalia but also the hypothalamic-pituitary axis.

Regarding the brain function and sexual behavior, perinatal administration of TCDD (200–800 ng/kg) to pregnant Long-Evans rats exerted changes in expression level of NMDA receptor subunits 2A and 2B mRNAs in offspring on PND 49 but not on PND 5. This result suggests that maternal exposure to TCDD alters the expression of certain genes in the brain in adulthood but not in the neonatal period. In order to test the hypothesis that perinatal exposure to dioxins affects the neocortical function and expression of sexual behavior in adulthood, male rat copulation after puberty was tested in perinatal TCDD-exposed male offspring. We found that the perinatal TCDD exposure significantly reduced the number of mounts and intromissions in male rats and suppressed the upregulation of brain-derived neurotrophic factor (BDNF) mRNA in the frontal cortex but not that of c-fos mRNA in the preoptic area (POA) . No change in the size of the POA was found. These results suggest that a relatively low dose of TCDD affects neocortical function, perhaps independent from brain sexual differentiation and alters the expression of sexual behavior[5,6].

Critical Period

It has been reported that there have been critical periods for the effects of TCDD. We found that windows for the critical period are open at specific time during gestation or lactation, depending

upon the endpoints of interest. Typical examples include male reproductive development, thyroid hormone homeostasis and immune function.

Regarding male reproductive organs, we administered TCDD (1 μg/kg) to pregnant Sprague-Dawley rats on either GD 15 or 18, and to male pups on PND 2, and sacrificed the offspring on PND 70. The administration of TCDD on GD 15 showed significant decreases in sperm numbers in the epididymis, prostate weight, and anogenital distance, which were not observed in the rats exposed to TCDD on GD 18 or PND 2. These results suggest that only *in utero* exposure determines the expression of a particular battery of genes that are responsible for the development of male reproductive organs, such as penis and prostate gland, later in adulthood[7].

The disruption of thyroid hormone homeostasis is affected predominantly by lactational exposure to TCDD, as shown by a cross-fostering experiment. Pregnant Holtzman rats were administered either TCDD or corn oil on GD 15, and pups were divided into 4 groups on PND 1: pups not exposed by either route, pups exposed only *in utero*, pups exposed only lactationally and pups exposed by both routes. When the pups were examined on PND 21, the two groups of rat offspring that were exposed to TCDD lactationally showed a significant decrease in serum total thyroid hormone concentrations and an increase in UDP-glucuronosyl transferase 1A6 and 7 as well as CYP1A1 in the liver. These mice showed hyperplasia of follicular cells in the thyroid on PND 49 [8].

Another interesting observation is the response of T-cell related immune function. When female C57BL/6 or Nc/Nga mice were exposed to TCDD (5 and 20 μg/kg) and ovalubumin (OVA) at 6 weeks of age, followed by OVA sensitization 3 weeks later, serum concentrations of antibodies including IgE as well as Th2-type cytokine production by splenocytes were suppressed in a TCDD dose dependent manner[9,10]. In contrast, when pregnant C57BL/6 mice were administered TCDD (0.3, 1.0 and 3.0 μg/kg) on GD12.5 and their female pups were sensitized with OVA at week 3 after birth, serum IgE and Th2-type cytokine production by splenocytes were elevated with 1.0 μg TCDD/kg (unpublished data). These results may allow us to speculate that allergy will be enhanced depending on the timing of TCDD exposure.

AhR dependent vs. independent toxicities

The use of AhR-null mice demonstrated that dioxins and aromatic hydrocarbons, such as benzo[a]pyrene, exert toxic effects including cleft palate, carcinogenicity and thymic involution in an AhR-mediated manner[11,12,13]. In the present study, we found that the following toxicities were also mediated in an AhR-dependent manner. First, wild-type male pups transplacentally exposed to TCDD showed a decrease of prostate weight gain accompanied by suppression of genes of marker proteins for prostate development, such as probasin, mp25 and PSP94, but AhR-null pups treated with TCDD did not show these effects [14]. Second, while TCDD-exposed wild-type mice showed significant disruptions in thyroid hormone and retinoid metabolism such as significant decreases in serum thyroid hormone and retinol concentrations as well as elevated levels of UGT1A6 and 7 mRNAs and their proteins in the liver (see Section B), no such alterations were found in AhR-null mice given TCDD[15].

To compare the effects of dioxn-like PCBs and non-dioxin like PCBs with TCDD, we administered PCB77 (50 mg/kg, based on the 1997 WHO TEF for PCB 77 of 0.0001, which is equivalent to 5 μg TEQ/kg) , PCB126 (1 mg/kg, based on TEF = 0.1, which is equivalent to 100 μg TEQ/kg), PCB153 (10 - 200 mg/kg, lacks TDCC-like activity) or TCDD (20 μg/kg) to 13-week-old C57BL/6 mice and examined thyroid hormone status 7 days later. As expected, CYP1A1 and UGT1A6 mRNA expression levels were increased by either TCDD or PCB126 administration. Although no significant increase in UGT1A6 was found by PCB77 or PCB153 administration,

serum total T4 concentrations were decreased in both treatment groups. It was found that serum total T4 concentration was significantly decreased in all treatment groups compared to the vehicle-treated group. The use of transthyretin-null mice suggested that PCB77 or perhaps its hydroxylated metabolite is at least partly responsible for this decrease. In the case of PCB153, the decrease in serum thyroid hormone concentration was dose-dependent and evident at a dose as low as 10 mg/kg. Other mechanisms rather than AhR or transthyretin are thought to be involved in the disruption of thyroid hormone homeostasis.

Difference in Susceptibility

A large difference in susceptibility to dioxin toxicity among animal species and strains has been accounted for at least in part by differences in the primary structure of AhR[16]. In the present research project, we have obtained two novel findings: strain differences in trans-placental fetal toxicity may be mediated by genes other than AhR, and sensitivity of humans might be less than even a dioxin-low sensitive mouse strain, DBA/2.

Regarding trans-placental TCDD toxicity, we found a degenerative change in glycogen cells, an increase in glycogen amount, and an elevated level of glucose transporter (GLUT) 3 mRNA in placenta and associated with an elevated incidence of fetal death (12.9% in 1.6 μg/kg group). These effects were dose-dependent when pregnant Holtzman rats were administered TCDD a single oral dose of 0.8 or 1.6 μg/kg on GD15 and examined on GD20 [17]. After the proteomics study using 2-D gel electrophoresis on the 15,000 x g supernatant fractions of placental homogenates, the elevated amounts of heat shock protein 27, β-tropomyosin, as well as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were found in TCDD-treated specimens. Uterine-ligation on GD15 resulted in a hypoxic condition and an increase in GAPDH, but not in other proteins on GD20. The histopathology as well as the proteomics study suggests that the increased incidence of fetal death may be due to hypoxia after TCDD exposure[18]. In sharp contrast, no such elevation of fetal death as well as pathological alteration was found in Sprague-Dawley rats even at a TCDD dose of 10 μg/kg. Interestingly, the aminoacid sequence of AhR of the both rat strains was found to be identical with each other and the dose-dependently induced pattern of CYP1A1 mRNA was similar in the two strains. These results suggest the presence of other modifying factors, maybe gene products, besides AhR.

Second, humanized AhR mice were produced by replacing murine AhR with human AhR in C57BL/6 mice in the hope that this transgenic mouse could mimic responses to TCDD in humans [19]. The response to TCDD was compared among mice strains, having either a high-affinity type AhR (AhR^{b-1}: C57BL/6 type), low-affinity type AhR (AhR^{d}: DBA/2type) or human AhR. The CYP1A1 gene expression level and the incidence of cleft palate were found highest in C57BL/6, intermediate in DBA/2, and lowest in humanized AhR mice. The present result suggests that humans may belong to the less sensitive animal species for certain endpoints of dioxin toxicity.

Conclusion

The CREST project provided new experimental evidence and confirmed earlier observations on: alterations of various endpoints by low dose TCDD exposure, the presence of critical windows for TCDD exposure to cause certain developmental effects, the presence of arylhydrocarbon receptor (AhR)-mediated or non-AhR-mediated toxicities and differences in TCDD sensitivities among various animal species and their strains. There is no doubt that experimental evidence is needed to provide the scientific basis for risk assessment.

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