Table 1: Pregnancy outcome and postnatal mortality of rhesus monkeys exposed to TCDD.

Group	No. of dams	No. of abortions	No. of stillborns	No. of live borns	No. of postnatal deaths	Gestation length (days)	Birth weight (g)
Control	23	2	3	18	5	161.8±7.8	426.1±58.6
30 ng/kg	20	0	5	15	3	163.8±5.9	426.8±56.9
300 ng/kg	20	2	2	16	8	164.9±9.7	408.6±63.7
300 ng/kg[1)]	9	5	1	3	1	165.0±3.0	466.0±87.1

1) Newly added group

Tooth abnormalities in the young: The incidence of tooth abnormalities in the young was shown in Table 2. Tooth abnormalities in the stillborn and postnatally died young were described previously[5]. No abnormalities were detected in the control and 30 ng/kg groups, whereas more than half of the young in the 300 ng/kg had tooth abnormalities as listed in Table 3. The upper permanent lateral incisors were most frequently affected. In contrast, among the deciduous teeth, the central incisors seemed to be most sensitive targets of developmental toxicity of TCDD. The permanent premolars were also affected frequently, while the canine and the first molar were resistant to the adverse effect of TCDD. Probably these larger teeth have become resistant to odontotoxic chemicals during the course of evolution.

Table 2: Incidence of tooth abnormalities among F1 exposed to TCDD.

Group	Stillborns and postnatally died young			Surviving young		
	No. of specimens	No. of specimens with tooth abnormalities (%)		No. of young	No. of young with tooth abnormalities (%)	
Control	4	0	(0)	13	0	(0)
30 ng/kg	5	0	(0)	12	0	(0)
300 ng/kg	8	3	(38)	8	6	(75)
300 ng/kg[1)]	2	0	(0)	2	1	(50)

1) Newly added group

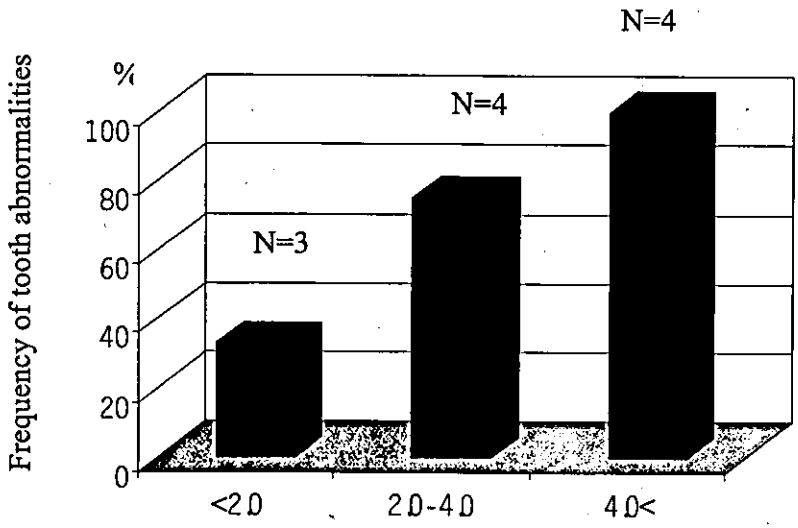
Relationship between maternal serum TCDD concentration and occurrence of tooth abnormalities: In the control maternal serum, the TCDD levels were below the detection limit. In the 30 ng/kg group, the levels were fairly constant, ranging from 0.19 to 0.21 pg/g wet weight. In contrast, the levels varied largely in the 300 ng/kg group, ranging from 1.1 to 8.9 pg/g wet weight. The average of those without tooth abnormalities in their young was 1.4 ± 0.6 pg/g wet weight, whereas that with tooth abnormalities was 4.3 ± 2.4 pg/g wet weight. The concentration-response relationship is shown in Fig. 1.

Table 3: Tooth abnormalities detected in the young exposed to TCDD at 300 ng/kg.

Young	Sex	Age (days)[2)]	Abnormal Findings
31	♀	1430	542|24 missing |5 conical
39	♂	1410	542|245 missing
42	♀	1415	5|5 missing |4 conical
44	♂	1415	54 |45 missing |5 conical
60	♂	1388	542|245 5|5 missing
66	♂	1338	52|2 1|1 missing
			54 |45 malaligned |45 conical
106[1)]	♀	688	A |A 4 |24 missing

1) Newly added group
2) Age at X-ray examination

Figure 1: Maternal serum concentration of TCDD and the incidence of tooth abnormalities.



Maternal serum concentration of TCDD (pg/g wet weight)
N = number of specimens in each concentration category

Validity of the current TDI: The above results indicate that the LOAEL body burden for induction of tooth abnormalities in the rhesus monkey is at a certain level between 30 ng/kg and 300 ng/kg, probably not much different from the LOAEL body burden for rodents, 86 ng/kg. Hence it is reasonable to conclude that the current TDI of dioxins in Japan needs no immediate modification.

Acknowledgements
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Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on signal transduction pathway-related protein expression in liver and cerebrum of rhesus monkey

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Introduction

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is known to produce a wide range of toxic and biochemical effects in experimental animals, including immunological dysfunctions, chloracne, tetragenecity and carcinogenesis[1-3]. Recently, the potential impact of dioxins on neurological disorders with particular focus on attention deficit hyperactivity disorder (ADHD) are concerned. Although a lot of information is available from studies in rodents[4-6], not much is known of the low dose effects of TCDD in non-human primates[7]. In higher animals, dioxins are metabolized slowly, as evidenced by the estimated TCDD half-life of 5.8 to 14.1 years[8]. Therefore, it is necessary to investigate the long-term effects of TCDD on human health. Considering the pronounced species differences observed in some studies of TCDD, the studies using primates are needed for assessment of TCDD exposure on human health. We have been studying the metabolism and the effects of single administration of TCDD on pregnant monkey (F0) and F1 rhesus monkey[9-11]. The focus of the present study is to study the effects of TCDD on signal transduction pathway-related protein levels in various organs, especially in liver and brain of F0 monkeys.

Methods and Materials

Chemicals and antibodies: 2,3,7,8-TCDD dissolved in toluene and DMSO (1:2, v/v) were purchased from Kanto Chemicals Co. Ltd. (Tokyo, Japan). Anti-aromatic (aryl) hydrocarbon receptor (Ah-R, 5579), anti-Ah-R nuclear translocator proteins (Arntl, 8076), anti-Akt1/2 (8312), anti-phospho-Akt1/2/3 (7985), anti-EGFR (03), anti-VE-cadherin (6458), anti-Bad (8044), anti-caspase3 (7148), anti-caspase8 (7890), and anti-beta-actin (1615) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-rabbit (7074) and mouse (7076) IgG, horseradish peroxidase-linked antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). An anti-cytochrome P450 1A1 (CYP1A1, 299124) was purchased by Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). Anti-goat IgG, horseradish peroxidase-linked antibody was obtained from Vector Laboratories, Inc. (Burlingame, CA, USA).

Animals: Rhesus monkeys were purchased from China National Scientific Instruments & Materials Import/Export Corporation (Beijing, China). All procedures involving animal care were in accord with the institutional guidelines in compliance with national laws. Monkeys (5-6 years old and 5.3-6.7 kg of body weight) were kept in Shin Nippon Biomedical Laboratories, Ltd. (Kagoshima, Japan). 2,3,7,8-TCDD (0, 30 and 300 ng/kg of body weight) was subcutaneously administered to pregnant monkeys (F0). The detailed breeding condition was described previously[12]. After delivery F0 monkeys have been observed for 3-4 years and sacrificed for analysis of protein and gene expression. In this study, the liver and cerebrum were obtained from 2 monkeys from three groups (0, 30 ng/kg TCDD, 300 ng/kg TCDD) which were observed for more than 3 years after TCDD administration.

Western blotting: The protein levels of various signaling transduction-related proteins were analyzed using western blotting. The proteins were visualized using Phototope®-HRP Western Blot detection system (7071, Cell Signaling Technology, Beverly, MA, USA). The level of proteins determined average of spot intensity per each proteins by using Chemidoc XRS system and Quantity One® image analysis software (Bio-Rad Laboratories, Inc., USA).

Results and Discussion

Effects of TCDD on protein levels: We observed alterations of signal transduction-related protein levels at more than 3 years after a single administration of low dose TCDD (0, 30 and 300 ng/kg) in liver and cerebrum of rhesus monkey (F0). The results analyzed by western blotting are expressed as an average of two monkeys, and summarized in Table 1 and Figure 1. Though TCDD did not alter the levels of Ah-R and Arntl in liver, TCDD (30 and 300 ng/kg) increased the level of CYP1A1 in the liver. In the cerebrum, there is no significant difference of CYP1A1 protein levels among control, 30 and 300 ng/kg of TCDD groups.

There were significant decrease of VE-cadherin protein levels in liver and cerebrum of TCDD-treated monkeys. As compared to untreated controls, VE-cadherin protein levels in liver were decreased 0.38-fold, and 0.46-fold in 30 ng of TCDD/kg group, and 300 ng of TCDD/kg group, respectively. In cerebrum, 300 ng of TCDD/kg decreased VE-cadherin protein level 0.45-fold, compared to the control group.

Epidermal growth factor receptor (EGFR) protein levels in liver and cerebrum were increased in 30 ng TCDD/kg-treated monkeys. There was no difference between the control and the 300 ng of TCDD/kg groups. As TCDD was reported to cause alterations in the growth factor signal transduction pathways in endocervical cells from single exposure to TCDD (2-4 μg/kg) in monkey[13], it is possible that 300 ng TCDD/kg caused down-regulation of EGFR protein levels.

The increase of Akt protein level, and phosphorylation of Akt, in liver and cerebrum were observed in 300 ng TCDD/kg-treated monkeys. The protein levels of Bad were significantly increased 2.7-fold in the liver and 1.8-fold in the cerebrum of TCDD (300 ng/kg)-treated groups. Akt plays a critical role in controlling the balance between survival and apoptosis[14], and Akt promotes cell survival by inhibiting apoptosis through inactivation of Bad[15]. In the liver of 300 ng of TCDD/kg -treated monkeys, caspase 8 protein levels were increased. Caspase 3 was also increased in cerebrum of 300 ng of TCDD/kg-treated monkeys. Bad, caspase 8 and caspase 3 are known to play roles in apoptosis signal transduction pathway

There are few reports which investigated the effect of TCDD on signal transduction-related protein levels in liver and cerebrum of rhesus monkeys. The

results in this study suggest that a single administration of low dose TCDD may induce apoptosis in liver and cerebrum. To elucidate whether TCDD induces apoptosis and carcinogenesis, we are currently studying the effects of TCDD at molecular level. This study is ongoing, and we will observe the effects of a single administration of low dose TCDD on hepatic dysfunctions and neurological abnormalities in rhesus monkeys.

Acknowledgements

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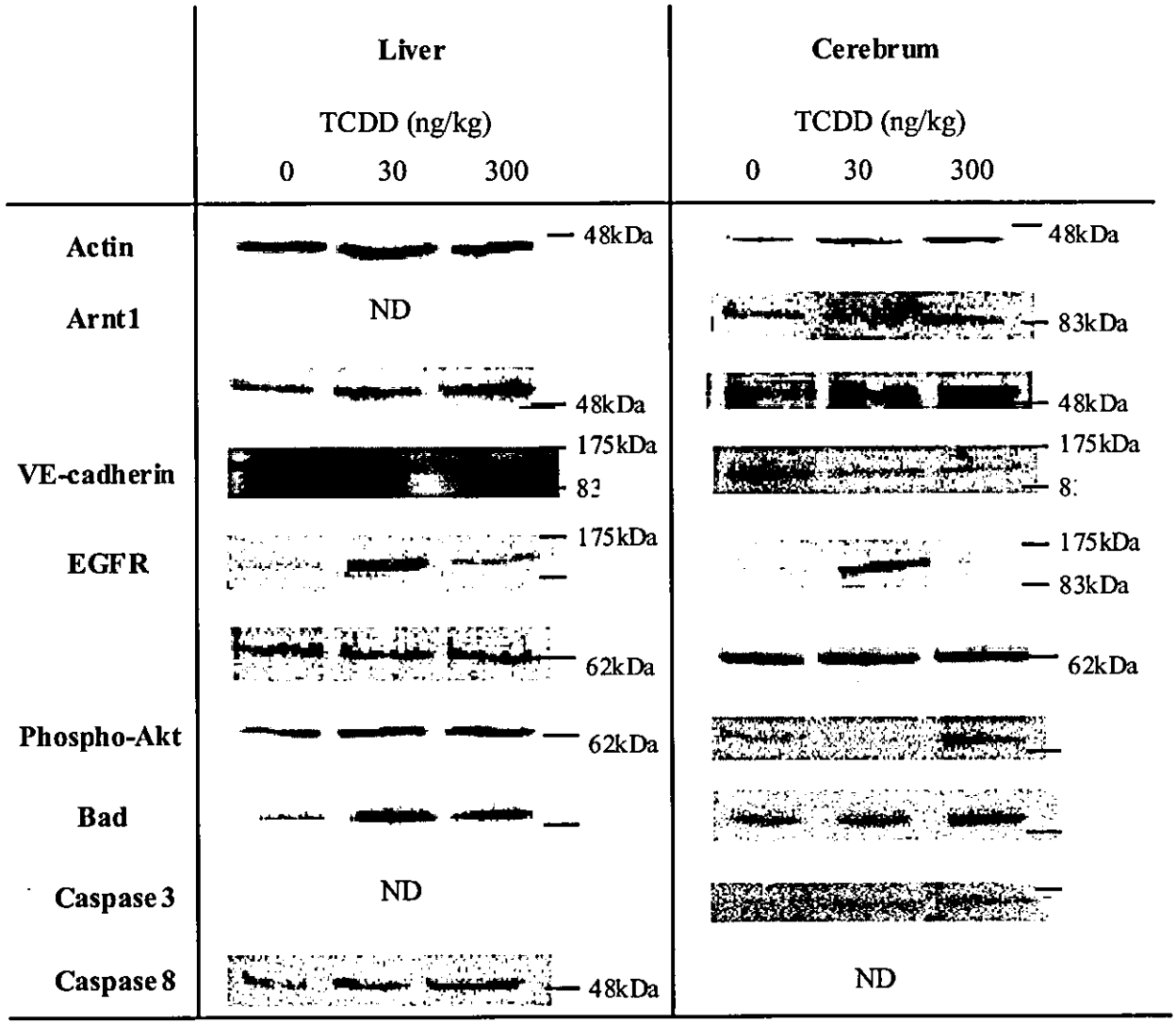
Table 1: Effects of TCDD on signal transduction pathway-related protein levels in liver and cerebrum of rhesus monkeys.

	Dose of TCDD (ng/kg)					
	0	30	300	0	30	300
	Liver			*Cerebrum*		
Ah-R		ND			ND	
Arntl		ND		0.084	0.112	0.177
CYP1A1	0.265	1.181	1.812	0.097	0.144	0.098
VE-cadherin	0.242	0.093	0.111	0.031	0.030	0.014
EGFR	0.084	0.161	0.112	0.032	0.091	0.050
Akt1/2	0.161	0.161	0.160	0.109	0.125	0.110
Phospho-Akt	0.214	0.281	0.439	0.047	0.061	0.070
Bad	0.052	0.101	0.140	0.126	0.225	0.227
Caspase3		ND		0.013	0.010	0.043
Caspase8	0.031	0.029	0.059		ND	

The results are shown as an average from two monkeys of each group (0, 30 ng/kg, and 300 ng/kg). The intensity of each protein per spot intensity was quantitated using Chemidoc XRS system and Quantity One® image analysis software (Bio-Rad Laboratories, Inc., USA), and corrected using the intensity of beta-actin protein level. ND represents not detected.

Figure 1: Effect of 30 and 300 ng of TCDD/ kg on protein levels liver and cerebrum.of rhesus monkeys

The expression levels of proteins in liver and cerebrum of TCDD-treated rhesus monkeys were determined by Western blotting. The beta-actin protein levels were used as controls confirming that an equal amount of protein was loaded.



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Liver injury in Rhesus monkeys subcutaneously injected with 2.3.7.8-tetrachlorodibenzo-p-dioxin

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Introduction

2.3.7.8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the most toxic member of dioxins which are environmentally and biologically stable. Exposure to these compounds results in wide variety of effects including immunological dysfunction, tetragenecity and carcinogenesis[1-4]. The liver is one of the central organs in which TCDD metabolized after absorption into the human and animal bodies. In experiments using rodents, TCDD accumulats and remains stable in the fatty tissues and liver for a long time. Kinetic profile of TCDD in our experiments using rhesus monkeys demonstrated the higher concentrations of TCDD in the fat, liver, and mammary gland[5-7]. TCDD-induced liver injury in humans has been reported in Japan (PCB), Taiwan (PCB or PCDF), Italy (Sebeso, TCDD), and Vietnam (TCDD). Considerating the pronounced difference between species observed in some studies on non-human primates to assess effects of relatively low dose of TCDD, in the present study, liver injury in rhesus monkeys after a single subcutaneous administration of low dose of TCDD during pregnancy was investigated.

Materials and methods

Chemicals: 2,3,7,8-TCDD dissolved in toluene and DMSO (1:2, v/v) were purchased from Kanto Chemicals Co. Ltd. (Tokyo, Japan).

Animals: Rhesus monkeys were purchased from China National Scientific Instruments & Materials Import/Export Corporation (Beijing, China). All procedures involving animal care were in accord with the institutional guidelines in compliance with national laws. 30ng/kg or 300ng/kg of TCDD was subcutaneously administrated to female pregnant monkeys. Control monkeys were administered vehicle alone. Three years after administration, ten monkeys (3, 4, 3 in each group) were sacrified. Macroscopic and histological studies of the liver followed by electron microscopic examination were carried out.

Immunohistochemstry: Immunohistochemical staining for MIB-1 (Dako cytomation, Glostrup, Denmark) as a proliferating cell marker and alpha smooth muscle actin (SMA) (Dako Cytomation, Glostrup, Denmark) as satellite cell marker was carried out. Monkey liver was fixed in 10% neutral-buffered formalin and embedded in paraffin for histological analysis. Immunohistochemical staining was performed on paraffin-embedded tissues using LSAB Kit (Dako Cytomation, Glostrup, Denmark) and DAB substrate kit (Nichirei, Tokyo, Japan). Monkey tissue sections (4 μm) were deparaffinized and rehydrated, and antigen retrieval was performed by treatment of the slides in 0.01M citrate buffer (pH 6.0) for 15 minutes in the microwave oven. Thereafter the slides were cooled to room temperature and washed in the phosphate buffered saline (PBS). Slides were immersed in 1% hydrogen peroxide for 30 minutes to block endogenous peroxidase activity. After washing and blocking, the sections were incubated at 4°C overnight with anti-MIB-1 antibody (Dako Cytomation, Glostrup, Denmark), followed by a standard procedure using biotin-blocking kit (Dako Cytomation, Glostrup, Denmark), LSAB kit and DAB-substrate kit. In the case of immunohistochemical staining with anti-alpha-SMA antibody, pretreatment with microwave oven was omitted. Incubation with anti-alpha-SMA antibody was performed at room temperature for 1 hour.

Western blot analysis: Liver tissue samples for protein analysis were frozen at once and kept at –80°C until use. The cells and tissues were homogenized in the

PBS containing 1mM EDTA, 0.2 mM PMSF and 1 μM pepstein A. Protein extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel (7.5%) electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane by electroblotting (150mA, at room temperature for 1h). Nonspecific binding of proteins was blocked by incubating the membranes in 5% non-fat milk in PBS containing 0.1% Tween-20. The membranes were incubated with anti-AhR (aryl hydrocarbon receptor) or anti-Arntl(aryl hydrocarbon receptor nuclear translocator 1) antibody (Santa Cruz Biotechnology, INC, CA) at 4°C overnight. The membranes were incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin antibody for 1 hour at room temperature. Detection of proteins which bind to primary antibody was performed with enhanced chemiluminscence reaction. (Amersham Biosciences Corp., Piscataway, NJ)

Results

Histopathological findings: Focal fatty change localized at the periphery and infarction with hemorrhage (Fig. 1) was found in 4 and 2 monkeys, respectively, to which TCDD was administrated (Table 1). Focal fatty change was nodular and simulated tumor. Coagulation necrosis or cytolytic change and hemorrhage were indicated in the infarction and infarctoid lesions of the liver. Parenchymal hemorrhage, sinusoidal ectasia and intrasinusoidal microthrombi-formation were also disclosed in 2, 5 and 4 monkeys, respectively. These abnormal histological findings were not found in control group of monkeys. Small cell hypercellularity of hepatocytes in the hepatic lobules was evident in 5 of 7 monkeys injected with TCDD.

Electron microscopic study Electron microscopic examination showed sinusoidal endothelical cell injury with degeneration and sinusoidal luninal stenosis.

Immunohistochemical staining of the liver: In control group, none has positive cells for alpha-SMA antibody. In contrast, intrasinusoidal alpha-SMA-positive cell hyperplasia was detected in most TCDD-administrated monkeys indicating satellite cell hyperplasia or transformation into the myofibroblast cells in TCDD-injected group. Small hepatocyte hypercellularity within hepatic lobules showed no labeling with MIB-1 antibody.

Western blot analysis of liver tissues: Control monkey has positive band at 110kD which corresponds to molecular weight of AhR. TCDD-injected ones have no positive band at the same position. On the other hand, positive bands as Arntl were observed both control and TCDD groups.

Discussion

These histopathological findings found in the liver of monkeys which were administrated with TCDD suggest sinusoidal endothelical cell injury and impairment in intrasinusoidal microcirculation because infarction, focal fatty change, and microthrombi-formation, that are rare events in the liver[8], are considerated to be closely associated with intrahepatic circularatoy impairment. As the hepatic parenchyma is protected against ischemia by its double blood supply, hepatic infarction is an uncommon lesion and usually accompanied by impairment in hepatic arterial and portal blood supply. Focal fatty change[8] is also unusually identified lesions which are first described in human in 1980 and becomes increasingly recognized by imaging techniques. It is surprising that these rare lesions were thus frequently identified in the liver of TCDD-treated rhesus monkeys. It is possible that small hepatocyte hypercellularity and alpha-SMA-positive satellite cell hyperplasia or transformation result from intralobular circulatory disturbance with local hypoxia and ischemic injury. Increased number of alpha-SMA-positive cells may suggest perisinusoidal fibrosis in the liver. There is no previous report describing these finding in the liver after injection with TCDD into the animal models although it has been reported that TCDD induced endothelical cell injury. It remains unclear whether or how TCDD induced intrasinusoidal endothelical cell injury. Further studies are necessary to explore the mechanism.

Acknowledgment

This study was supported by Health Science Research Grants for Research on Environmental Health from the Ministry of Health and Welfare of Japan.

Figure 1. Hemorrhagic infarction of the liver detected in rhesus monkey injected with TCDD.

Table 1. Histopathological findings in the liver of TCDD- administrated rhesus monkeys.

	Positive /total number examined in monkeys injected with TCDD		
	Control	30ng/kg	300ng/kg
Focal fatty change	0/3	2/4	2/3
Fatty change	0/3	1/4	1/3
Infarction	0/3	1/4	1/3
Hemorrhage	0/3	1/4	1/3
Microthrombi	0/3	2/4	2/3
Sinusoidal ectasia	0/3	3/4	2/3
Small cell hypercellularity	0/3	2/4	3/3
Alpha-smooth muscle actin-Positive cell hyperplasia	0/3	3/4	3/3

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In utero and lactational exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) affects tooth development in rhesus monkeys

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In utero and lactational exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) affects tooth development in rhesus monkeys

Abstract

We thought to validate the current tolerable daily intake (TDI) value for dioxin (4 pg/kg) in Japan. Pregnant rhesus monkeys received an initial dose of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 0, 30, or 300 ng/kg subcutaneously) on day 20 of gestation; the dams received additional injection of 5% of the initial dose every 30 days until day 90 after delivery. The teeth of stillborn, postnatally dead, and surviving offspring (now approximately 4 years old) were evaluated. None of the offspring in the 0- and 30-ng/kg groups (n=17 and 15, respectively) had tooth abnormalities, whereas 10 of 16 in the 300-ng/kg had them. These findings suggest the lowest-observed-adverse-effect-level (LOAEL) for TCDD in the rhesus monkey is between 30 and 300 ng/kg, and probably is close to that for rodents (86 ng/kg), on which the current TDI was based. It is reasonable to conclude that the current TDI needs no immediate modification.

Keywords: Dioxin; TCDD; Tooth; Rhesus monkey; Primate; TDI; Developmental toxicity; LOAEL

1. Introduction

Dioxins are ubiquitous environmental pollutants. Although contamination levels are decreasing [1], the adverse effects of dioxins, especially their reproductive and developmental toxicities, still attract much public concern, and regulatory agencies worldwide are seeking to define a reasonable permissible intake level. In Japan, the current tolerable daily intake (TDI) of dioxin and dioxin related compounds has been set at 4 pg toxic equivalent (TEQ)/kg/day [2]. This value was calculated from the lowest-observed-adverse-effect level (LOAEL) in experimental animals, mostly rodents. A single oral dose of 200 ng/kg of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) to pregnant rats on day 15 of gestation resulted in abnormalities of reproductive organs in the offspring [3]. The maternal body burden at this dose was measured to be 86 ng/kg. To attain this body burden level, human daily intake was calculated to be 43.6 pg/kg/day. An uncertainty factor of 10 was applied to this value, and the human TDI of 4 pg/kg was established. However, great differences between the biological half-life of TCDD in humans and rodents have called into question the validity of this calculation. To obtain a more reliable LOAEL for dioxins, in 1999 we initiated a long-term developmental toxicity study in rhesus monkeys.

In rodents, the teeth are known targets of the developmental toxicity of dioxin; *in utero* and lactational TCDD exposure affects incisor and molar development in rats [4]. Tooth abnormalities also occurred among human populations accidentally exposed to dioxins [5] or polychlorinated biphenyls (PCBs) and polychlorinated dibenzofurans (PCDF) [6,7]. During our monkey experiment, some offspring were stillborn or died neonatally. These animals provided us with a unique opportunity to study tooth development in primate offspring exposed to TCDD *in utero* and while nursing. Macroscopic observation revealed tooth abnormalities in the offspring from mothers exposed to a relatively high dose of TCDD (300 ng/kg on day 20 of gestatoion and 15 ng/kg every 30 days during pregnancy) [8]. This finding prompted us to examine surviving offspring radiographically, and we found that tooth abnormalities occurred at a high frequency in the high-dose group. These offspring are still alive and growing, and various studies are in progress. This report describes the dental findings obtained as of April, 2004.

2. Materials and methods

2.1. Animals

Colony bred adult female rhesus monkeys (age, 3-10 years; weight, 4-7 kg) were purchased from China National Scientific Instruments & Materials Import/Export Corporation (Beijin, China). Details of breeding conditions are given elsewhere [9]. Briefly, the animals were housed in stainless-steel cages (68 cm × 70 cm × 77 cm), and received approximately 144 g of solid diet (Harlan Tekland, Harlan Sprague Dawley Inc., Indianapolis, IN) daily. The rooms were maintained at 26±2℃ and 50±10% relative humidity and on a 12-h light cycle (lights on, 0600-1800 h). Female monkeys were allowed to cohabit with males on days 12, 13, and 14 of the menstrual cycle. When copulation was confirmed visually, the median day of the mating period (day13 of the menstrual cycle) was designated as day 0 of gestation (GD 0). On GD 18 or 19, pregnancy was confirmed by ultrasonography (SSD-2000, Aloka Co., Ltd, Tokyo, Japan) of animals anesthetized by an intramuscular injection of 5% ketamine hydrochloride (5-10 mg/kg, Sigma-Aldrich Corporation, St. Louis, MO). Pregnant monkeys were divided into three groups, each consisting of approximately 20 animals. During gestation, all dams were observed for general condition at least once daily and they were weighed once every 20 days.

The dams were allowed to deliver naturally. The day on which delivery was detected was designated as postnatal day 0 (PND 0). Delivered offspring were examined macroscopically, and allowed to cohabit with their mothers for approximately 1 year. The offspring were weighed once every 10 days until PND 90, once every 20 days until PND 150, and once every 30 days thereafter. The animals were reared in the monkey facility of Shin Nippon Biomedical Laboratories, Ltd. (SNBL, Kagoshima, Japan) and were treated humanely according to the guidelines of animal experiments for SNBL. Animal excreta and carcasses were handled with extreme care, and all waste was burned in an incinerator equipped with an afterburner held at >800℃.

2.2. Chemicals and administration

TCDD (lot number 110899, purity >98% as determined by gas chromatography, Wellington Laboratories Inc., Guelph, Ontario, Canada) was dissolved in a mixture of toluene/dimethylsulfoxide (DMSO; 1:2, v/v) at a concentration of 300 ng/ml. The solution was prepared by Kanto Kagaku Co., Ltd. (Tokyo, Japan) and final concentrations were confirmed by gas chromatography. Confirmed pregnant female monkeys received TCDD subcutaneously into the

back region on GD 20 at an initial dose of 30 or 300 ng/kg. This route was selected to avoid uncertainty of absorption by oral administration. The dosing volume was 0.1 ml/kg for the lower-dose group and 1 ml/kg for the higher-dose group. Controls received the vehicle in a volume of 1 ml/kg. To maintain the desired body burden, dams received 5% of the initial dose (i.e. 1.5 or 15 ng/kg) every 30 days during pregnancy and lactation until PND 90. For the maintenance dosing, a TCDD solution at a concentration of 30 ng/ml was prepared, and animals in the lower-dose group received 0.05 ml/kg in each injection whereas those in the higher-dose group received 0.5 ml/kg. The total dose administered to the higher-dose group was 405 (300 + 15 × 7 for dams with gestation length less than 170 days) ng/kg, or 420 (300 + 15 × 8 for dams with gestation length 170 days or more) ng/kg, and that to the lower-dose group was 40.5 or 42 ng/kg. The lower dose level was set at about one third of the LOAEL body burden in rodents (86 ng/kg) and the higher one at about three times the LOAEL. The maintenance dosing schedule was set according to the assumption that the biological half-life of TCDD in rhesus monkeys is approximately 1 year [10].

2.3. Macroscopic observation

Stillborn fetuses and offspring that died by PND 100 were necropsied, and the upper and lower jaws were dissected for detailed observation. Macroscopic observation was made under a dissecting microscope (SZX12, Olympus Corporation, Tokyo, Japan). Photographs were taken using a digital camera (C-4040, Olympus). Surviving offspring were anesthetized by intramuscular injection of ketamine at 10 mg/kg into the thigh before intraoral examination, and photographs were taken using an intraoral digital camera (Crystal Cam II, GC Co., Ltd., Tokyo, Japan).

2.4. Radiographic observation

Conventional intraoral radiographs were taken using a portable X-ray apparatus (KX-60, Asahi Roentgen Ind. Co., Ltd., Kyoto, Japan) with a charge coupled device (CCD; Gendex Visualix, Dentsply International Inc., York, PA).

2.5. Statistical analysis

All the data were analyzed using JMP5.1.1J (SAS Institute Japan, Tokyo, Japan). Analysis of variance was used to compare measurement data such as length of gestation and body weight. The incidence of tooth abnormalities was compared by using Fischer's exact probability test. A statistically significant difference was confirmed at $P < 0.05$.