DEFECT OF MINERALIZATION AS A POSSIBLE CAUSE OF 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN–INDUCED BONE TOXICITY IN EARLY POSTNATAL DEVELOPMENT IN MICE

Noriko Nishimura[1], Tomohiro Ito[1], Hidekazu Fujimaki[1] and Hisao Nishimura[2]

[1]Research Center for Environmental Risk, National Institute for Environmental Studies, Tsukuba, 305-8506 Japan; [2]Human Sciences, Aichi Mizuho University, Toyota, 470-0394 Japan

Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a notorious environmental hazard that causes acute and chronic toxicity, including thyroid toxicity, teratogenicity, carcinogenicity, and developmental defects in laboratory animals[1,2]. The available data in animals and humans further suggest that bone could be another target tissue for TCDD. Bone homeostasis is maintained by a balance between bone resorption by osteoclasts and bone formation by osteoblasts. An imbalance in the bone remodeling process results in clinical diseases of the skeleton such as osteoporosis or osteopetrosis. Human epidemiologic studies showed impaired development of teeth by perinatal dioxin exposure in Yucheng children[3] as well as mineralization defects of the molars in children[4]. In laboratory animals, the toxic effects of TCDD on bone have indicated that earlier exposure caused more severe bone defects. Furthermore, lactational exposure to TCDD covering the early phases of bone development is required to cause severe bone defects[5]. We therefore used mice exposed to TCDD through lactation as animal models in this study.

The main objective of this study was to identify the molecular mechanism underlying the TCDD-mediating bone toxicity by examining the expressions of bone turnover-associated genes and bone histology as well as biochemical markers of bone remodeling in serum of mice.

Materials and methods

Animals and treatments

After spontaneous delivery, dams were given oral TCDD (15 μg/kg body weight) or an equivalent volume of corn oil as vehicle on postnatal day (PND) 1, and pups were exposed to TCDD via lactation. Serum and tissues from pups were collected on PNDs 7, 14, and 21 and analyzed.

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the kidney and tibia using TRIzol reagent, and cDNA was synthesized.

mRNA of various genes including osteocalcin, receptor activator of NF-κB ligand (RANKL), receptor activator of NF-κB (RANK), and osteoprotegerin (OPG) were determined with a LightCycler (Roche Diagnostics, Mannheim, Germany) using the Fast Real-Time SYBR Green PCR Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Micro-x-ray computed tomography (μCT) analyses of the tibia

Tibias obtained from 21-day-old mice were dissected free of soft tissue. The bones were subjected to μCT analysis. In the present study, cancellous bone parameters were measured at a site that was 2-10 mm distal to the lower margin of the growth plate in the proximal tibial metaphysis, which consists of secondary spongy bone.

Histomorphometric analysis of bone

Tibias obtained from 21-day-old mice treated with either TCDD or vehicle oil were removed, dissected free of tissue, fixed immediately in 70% ethanol, and embedded in methyl methacrylate resin without decalcification (Wako, Tokyo, Japan). Sections, 5-μm sections were stained with Villanueva's Goldner to discriminate between mineralized and unmineralized bone (osteoids); quantitative histomorphometry was analyzed by an image analysis system with the analysis software (Osteoplan). The histomorphometric parameters of bone were calculated as defined by Parifitt et al[6]. The following parameters of cancellous bone were measured: total tissue volume (TV), bone volume (BV), bone surface (BS), and eroded surface (ES). These data were used to calculate the percent cancellous bone volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and the percent eroded surface (ES/BS). Tartrate-resistant acid phosphatase staining was used to identify osteoclasts in frozen sections. The number and surface area of osteoclasts per bone surface area (N.Oc/BS and Oc.S/BS, respectively) were measured.

Results

Effect of TCDD on gene expression related to osteoclastic bone resorption in tibia

The RANKL/RANK signaling system plays crucial roles for osteoclastogenesis and activation of mature osteoclasts leading to bone resorption. OPG prevents this interaction by competing binding to RANKL, resulting in inhibition of osteoclast formation and activation of mature osteoclasts. These gene expressions were unaffected by TCDD.

Effect of TCDD on gene expression of osteocalcin and alkaline phosphatase in serum and tibias

TCDD did not affect serum levels of osteocalcin on PND 7, but the agent did decrease osteocalcin concentrations to about 70% of the control level on PND 21. Because serum osteocalcin levels were significantly reduced by TCDD, we next assessed the effects of TCDD on mRNA expression of tibial osteocalcin and alkaline phosphatase, which are known to be the most commonly used markers of

osteoblastic bone formation. mRNA levels of osteocalcin and alkaline phosphatase in tibias were significantly decreased to about 50% of the control level.

Bone histomorphometry and histological analysis of bone

Bone mineral density, cortical thickness, and bone mineral content were all decreased significantly in TCDD-exposed pup tibias. Although decreased gene expression of markers of the osteoblastic bone formation suggested the toxicity by TCDD might be produced through an inhibition of osteoblastic activity, we next determined the possibility that an increase in osteoclastic bone resorption is responsible for TCDD-induced bone toxicity by histomorphometric and histological analyses. Whereas there was no difference in TRAP-positive osteoclast numbers and ES/BS between the TCDD-treated and oil-treated mice, bone formation-related parameters such as osteoid volume/bone volume, and osteoblast surface/bone surface were all significantly higher in TCDD-exposed mice than in oil-treated control mice. Bone histology with Villanuevas's Goldner staining revealed the dramatic increase in the amount of unmineralized osteoid on the periosteal surface at the proximal tibial metaphysis and on trabecular surfaces as well as the dramatic reduction in mineralized bone in the tibia of the TCDD-exposed mice. Of particular note was the severely reduced amount of bone in the tibia of TCDD-treated mice in contrast to oil-treated control mice with regular morphology with osteoid seams lining the trabecular bone surface.

Discussion

In the present study, we showed the toxic effects of TCDD on bone development with μCT analysis. Our RT-PCR analyses revealed that gene expressions participating in osteoclastic bone resorption were not affected by TCDD. In addition, the bone histomorphometry study demonstrated an unchanged number of osteoclasts and ES/BS. These results indicate strongly that the activation of bone resorption was not primarily responsible for the TCDD-induced bone toxicity.

We thus analyzed a possible involvement of TCDD to suppress the osteoblastic bone formation activities as an alternative mechanism by measuring biochemical markers of bone formation. The most striking finding from the current study is that in addition to alkaline phosphatase expression, exogenous administration of TCDD significantly inhibited expression of osteocalcin at both the mRNA and serum protein levels. It is reasonable to speculate that this striking reduction of osteocalcin expression may have a causative role in TCDD-induced bone toxicity because osteocalcin is the molecular determinant of bone formation via the contribution to mineralize bone. Our current observations clearly showed that TCDD inhibited bone formation activities. In addition, TCDD-induced impairment of bone mineralization was proven by histological examinations with Villanuevas's Goldner staining. The marked increase in the amount of unmineralized osteoid as well

as the dramatic reduction in mineralized bone in the tibia of the TCDD-exposed mice was characteristic toxic lesions. Of particular note was the severely reduced amount of bone accompanied with morphologically abnormal bone structure in TCDD-treated tibia in contrast to a tissue with a regular morphologic arrangement of oil-treated control mice. Based on these lines of evidence, it may be concluded that bone toxicity by TCDD might be induced via suppression of the osteoblastic bone formation rather than promotion of osteoclastic bone resorption, which leads to the impairment of bone mineralization.

References

1. Pohjanvirta R. and Tuomisto J. *Pharmacol Rev* 1994; 46: 483.
2. Nishimura N., Yonemoto J., Miyabara Y., Sato M. and Tohyama C. *Endocrinology* 2003; 144: 2075.
3. Wang S.L., Chen T.T., Hsu J.F., Hsu C.C., Chang L.W., Ryan J.J., Guo Y.L. and Lambert G.H. *Environ Res*. 2003; 93: 131.
4. Alaluusua S., Lukinmaa P.L., Torppa, J., Tuomisto J. and Vartiainen T. *Lancet* 1999; 353: 206.
5. Miettinen H.M., Pulkkinen P., Jamsa T., Koistinen J., Simanainen U., Tuomisto J., Tuukkanen J. and Viluksela M. *Toxicol Sci* 2005; 85: 1003.
6. Parifitt A.M., Drezner M.K., Glorieux F.H., Kanis J.A., Malluche H., Meunier P.J., Ott S.M. and Recker R.R. *J Bone Miner Res* 1987; 2: 595.