TCDD disrupts posterior palatogenesis and causes cleft palate

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ABSTRACT

Dioxins (e.g. 2,3,7,8-tetrachlorodibenzo-p-dioxin; TCDD) cause cleft palate at a high rate. A post-fusional split may contribute to the pathogenesis, and tissue fragility may be a concern. The objective of this study was to investigate the effects of TCDD on the palatal epithelium, bone and muscle, which contribute to tissue integrity.

ICR mice (10−12 weeks old) were used. TCDD was administered on E12.5 at 40 mg/kg. Immunohistochemical staining for AhR, ER-α, laminin, collagen IV, osteopontin, Runx2, MyoD, and desmin were performed. Furthermore, western blot analysis for osteopontin, Runx2, MyoD, and desmin were performed to evaluate protein expression in the palatal tissue.

Immunohistologically, there was little difference in the collagen IV and laminin localization in the palatal epithelium between control versus TCDD-treated mice. Runx2 and osteopontin immunoreactivity decreased in the TCDD-treated palatal bone, and MyoD and desmin decreased in the TCDD-treated palatal muscle. AhR and ER-α immunoreactivity were localized to the normal palatal bone, but ER-α was diminished in the TCDD-treated palate. On western blot analysis, Runx2, MyoD, and desmin were all downregulated in the TCDD-treated palate.

TCDD may suppress palatal osteogenesis and myogenesis via AhR, and cause cleft palates via a post-fusional split mechanism, in addition to a failure of palatal fusion.

1. Introduction

Cleft lip and palate is one of the most common birth defects (Poswillo, 1989; Pausch et al., 2012). The pathogenesis of isolated cleft palate has been reported to be a different mechanism from cleft lip and/or palate (Ferguson, 1988; Yamada et al., 2005). A variety of causes of cleft palate have been reported (Vanderas, 1987; Natsume et al., 2000), involving genetic and environmental factors (Zhang et al., 2011). 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a member of the halogenated aromatic hydrocarbons, is a widespread environmental contaminant (Okumura et al., 2004). TCDD causes cleft palate at high rates in mice (Pratt et al., 1984; Weber et al., 1985; Abbott and Birnbaum, 1989a,b, Abbott et al., 1999; Yamada et al., 2006). Our previous studies revealed that TCDD is a strong teratogen for cleft palates, but has no effect on cleft lips in mice (Yamada et al., 2006). One leading hypothesis is that TCDD inhibits the programmed cell death of the medial edge epithelial (MEE) cells in the palatal shelves (Abbott and Birnbaum, 1989a,b; Abbott et al., 1999), and another hypothesis suggests that TCDD suppresses the mesenchymal growth of the palatal shelves (Takagi et al., 2000). The authors reported that the palates of some fetuses ruptured after both palatal shelves had fused (Yamada et al., 2006, Imura et al., 2010). This post-fusional split phenomenon may be applicable to humans (Kitamura, 1991), and tissue fragility may be a concern with this mechanism.

In this study, several factors responsible for tissue integrity were analyzed to elucidate the mechanisms of cleft palate induction by TCDD.

2. Material and methods

2.1. Animals and TCDD treatment

Pregnant ICR strain mice, 10−12 weeks of age, were obtained from Japan SLC Inc. (Shizuoka, Japan). The day of vaginal plug confirmation was defined as embryonic day 0.5 (E0.5). E12.5 pregnant female mice were then treated with TCDD (AccuStandard Inc., New Haven, CT) in olive oil with a gastric tube at 40 μg/kg, which cause cleft palate in

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100% of the fetuses (Yamada et al., 2006). Control animals were given an equivalent volume of the vehicle alone. Animal care conformed to the Guidelines for Animal Research of Kochi University in accordance with the Helsinki Declaration. The study protocol was approved by the Animal Care and Ethics committee of Kochi University.

2.2. Immunohistochemistry

The palates for histological examination were fixed overnight at 4 °C in 4% paraformaldehyde, dehydrated through an ethanol series, embedded in paraffin, and cut into 6 μm sections. The sections were incubated with 0.03% H_2O_2 for 5 min to block non-specific peroxidase activity. The sections were then washed with phosphate-buffered saline (0.1 M; PBS) three times for 5 min each and incubated with the primary antibody overnight on ice. Specific affinity-purified rabbit polyclonal antibodies directed against AhR (Santa Cruz, Delaware, CA), ER-α(Santa Cruz), osteopontin (Abcam, Cambridge, MA), and desmin (Abcam), and a mouse monoclonal antibody directed against Runx2 (Abcam) and MyoD (Sigma–Aldrich, Co., St. Louis, MO) were purchased as primary antibodies. When using the mouse monoclonal antibodies, Mouse Ig Blocking Reagent (Vector Laboratories, Inc., Burlingame, CA) was used to block the intrinsic immunoglobulins, prior to incubation with the primary antibodies. The primary antibodies were visualized using an EnVision+ System HRP (DAKO, Carpinteria, CA) kit.

The sections were washed with PBS three times for 5 min each and subsequently incubated for 30 min with the labeled polymer. The sections were then washed with PBS for 3 × 5 min and incubated with the 3,3′-diaminobenzidine (DAB) substrate-chromagen solution for 5–10 min and rinsed in distilled water. The sections were counterstained with 0.4% hematoxylin to provide contrast. At least three specimens were immunostained by each antigen.

For type IV collagen and laminin immunostaining, after incubating with anti-laminin and type IV collagen polyclonal antibodies (Sigma), the sections were incubated with Alexa Fluor-594 goat anti-rabbit IgG for immunofluorescence. The sections were then examined under an All-in-one Type Fluorescence Microscope (BZ-8000; Keyence, Osaka, Japan) using BZ Analyzer Software (Keyence).

2.3. Western blot analysis

After TCDD or vehicle alone treatment at E12.5, the dams were killed on E15.5, and the embryos were collected. The palatal shelves were dissected to minimize contamination by the tooth buds. Next, the palates were carefully divided into three parts; the anterior half of the hard palate (anterior), the posterior half of the hard palate (middle), and the soft palate (posterior) under a stereoscopic microscope. The samples were homogenized, and the protein was extracted using T-PER Tissue Protein Extraction Reagent (PIERCE, Rockford, IL).

The extracted proteins (50 μg/lane) were separated by SDS–polyacrylamide gel electrophoresis and transferred onto an Immobilon-P membrane (Immobilon; Millipore Corp., Bedford, MA, USA). Blocking was performed in Tris-buffered saline containing 5% (w/v) skim milk powder and 0.1% (v/v) Tween 20. The membranes were probed with antibodies against Runx2 (Abcam), osteopontin (Abcam), desmin (Abcam), and MyoD (Sigma–Aldrich) at a 1:1000 dilution. Visualization was performed using the ECL system (Amersham, Piscataway, NJ, USA). Three independent experiments with different samples were performed, and the average ratio was calculated as the means of all three experiments.

2.4. Statistical analysis

All results are shown as means ± standard deviation (S.D.). Comparisons between two groups were performed using Student's t-test. All statistical analyses were performed using Excel Statistics 2008 software (SSRI Co., Ltd, Tokyo, Japan), and p values <0.05 were considered to be statistically significant.

3. Results

3.1. Post-fusional split of the posterior palate causes cleft palate in mice

Starting on E14, both palatal shelves start to fuse in normal ICR mice, and 100% of the palates are fused by E15. In the TCDD treated mice, palatal fusion was observed from E14 to E16 in 3–18% of the fetuses, but ultimately all palates did not fuse, and had separated by E18 (Fig. 1). This indicates that some of the palates had fused, but subsequently ruptured.

Representative palatal forms (A–C) and histological sections (D–F) of TCDD-exposed mice are shown in Fig. 2 (E15–E16). Some fetuses were confirmed to have completely fused palates (A, D). Some palates presented with a thin and sparse palatal midline (B), and seemed to be on the verge of rupture (E). Finally, all fetuses presented with cleft palates by E16 or later (C, F).

3.2. Mucosal basement membrane was normal in TCDD treated mouse palates

To estimate the epithelial integrity, the immunolocalization of type IV collagen and laminin was investigated. Both proteins were localized in the palatal epithelium in both control and TCDD treatment mice on E15 (Fig. 3).

3.3. Osteogenesis was suppressed in TCDD treated mouse palates

Runx2 and OPN are markers of osteoblast differentiation and osteogenesis (Yang et al., 2008; Ben-David et al., 2011; Behnia et al., 2012). Immunostaining for Runx2 and OPN (osteopontin) revealed that the localization of both proteins in the palatal bone had decreased following TCDD treatment on E16 (Fig. 4A, B).

On western blot analysis, Runx2 immunoreactivity was expressed more intensely in the anterior part of the palate than in the posterior. In the TCDD treated palate, both Runx2 and osteopontin were downregulated (Fig. 4C).
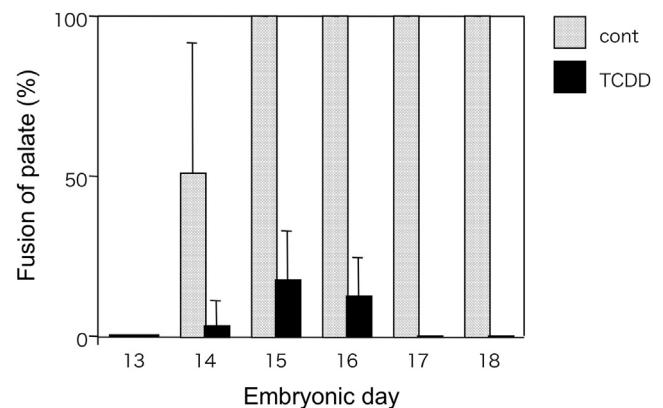
Fig. 1. Palatal fusion rate in normal and TCDD-treated mice. Starting on E14, both palatal shelves begin to fuse in normal ICR mice, and 100% of the palates had fused by E15. In TCDD treated mice, palatal fusion was observed from E14 to E16 in 3–18% of the fetuses, but all of the palates had separated by E18. The palates were randomly removed from three to five litters on each embryonic day (n = 95 (cont), n = 88 (TCDD)).