Cell Proliferation and Cell Death in the Medial Edge Epithelium during Palatogenesis and in the Developing Ureteral Epithelium in the Mouse Fetus: Pathogenesis of Cleft Palate and Hydroureter Induced by 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)

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1. Introduction

Cell proliferation and programmed cell death are important factors during palatogenesis. Secondary palatal shelves bulge from the maxillary processes and take a vertical position on each side of the tongue. They elevate to a horizontal position above the tongue. Medial edges of the horizontally rotated shelves come into contact each other and begin to fuse, forming the secondary palate. In the mouse (Jcl:ICR strain) the horizontal elevation and initial contact of shelves take place at gestational day (GD) 14.25 (vaginal plug = GD 0), and the fusion process proceeds until GD 15.0. The medial edge is covered with an epithelium, which is composed of a two-cell layer of medial edge epithelial (MEE) cells; basal cells lying on the basal lamina and peridermal cells facing the oronasal cavity. Prior to initial contact and adhesion, the peridermal cells undergo programmed cell death (PCD) and are shed. Basal cells of each palatal shelf are forced to come in contact. An midline epithelial seam is formed in the fusing palate. Epithelial cells in the seam undergo PCD and disappear[1]. The midline epithelial seam loses its continuity with the epithelium facing the oral and nasal epithelium and forms cell islands.

It is well known that dioxins induce cleft palate and hydronephrosis in the mouse fetus. Among dioxins 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is known as the most potent teratogen.

As for the pathogenesis of cleft palate, it has been suggested that TCDD does not interfere with growth, elevation, or initial contact of the palatal shelves, but does interfere with firm adhesion and/or degeneration of the medial epithelial cells[2]. It is reported that TCDD prevents programmed cell death of the medial peridermal cells and that the medial epithelial cells continue cell proliferation and differentiate into a stratified, squamous, keratinizing epithelium[3]. However, we showed that the MEE cells undergo cell death in the presence of TCDD and that PCD occurs as scheduled at the proper area as well as at the expected timing also in the TCDD group[4]. In the present study we examine whether TCDD induces active proliferation of MEE cells or not.

Hydronephrosis is also called hydroureter or dilated renal pelvis. It is reported that dioxins induce hyperplasia of the ureteral epithelium and obliteration of the ureter, resulting in hydronephrosis[5, 6]. Due to the obliteration, the renal pelvis is dilated and renal cortex and medulla become thin. However, description of normal development and cell turnover of the ureter is still lacking in the mouse fetus. In addition, ultrastructural aspects and cell turnover of the urothelium have not been reported in the adult mouse ureter. The aim of this study is to examine normal development and cell turnover of the ureter and to examine how TCDD induces hydroureter.

2. Materials and methods

Colony-bred Jcl:ICR mice from CLEA Japan, Inc. (Tokyo) were used. Mature females were mated with males overnight. Copulation was ascertained by the presence of a vaginal plug on the following morning, and the day on which the plug was found was designated as gestational day (GD) 0.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was used in this study. TCDD purchased from Cambridge Isotope Laboratories Japan (Catalog No. ED-901) was dissolved in corn oil. At GD 12.5 the dams were treated with TCDD at a dose of 40 μg/kg body weight (b.w.) by gavage. Untreated

dams were served as controls. The dams were killed by cervical dislocation at desired gestational days and the fetuses were harvested in phosphate buffered saline and fixed.

A. Observation of palatogenesis
Fetuses were taken out at GD 13.5, 14.0, 14.25, 14.5, 14.75, 15.0, or 15.5. For routine paraffin sections, the head was fixed with the mandible and tongue in Bouin's fixative or 10% formalin.

For detection of cell proliferation, immunohistochemistry with bromodeoxyuridine (BrdU) / anti-BrdU antibody was applied.

A–a) Immunohistochemistry with coronal paraffin sections
Dams were given an intraperitoneal injection of BrdU at 300 mg/kg b.w. 2 hr before sacrifice. This administration route was appropriate also for detection of BrdU-labeled cells in the fetus. Fetuses were fixed with 10% formalin for 12 hr and the head was embedded in paraffin. Serial sections (8 μm thick) from the nostril to the pharynx were incubated in 1 N hydrochloric acid for 20 min, in anti-BrdU monoclonal antibody (Amersham) for 1 hr, in peroxidase-labeled anti-mouse IgG2a for 30 min, and in a medium containing DAB and hydrogen peroxide.

A–b) Immunohistochemistry with a whole-mount specimen by confocal laser scanning microscopy (CLSM)
The head region after removal of the tongue and mandible was incubated in a medium containing BrdU (0.3 mg/ml medium) for 1 hr at 37°C, fixed in 10% formalin for 24 hr at 4°C, and permealized in 0.5% triton X-100 for 24 hr at 4°C. The head was bathed in anti-BrdU monoclonal antibody overnight at 4°C and BrdU-labeled cells were visualized in FITC-labeled anti-mouse IgG2a, and observed in a CLSM (Carl Zeiss LSM410). A palatal shelf was set so that the medial edge epithelium could be directly observed. Optically sectioned images were collected and reconstructed. This method facilitated to take an overview of the shelf and MEE cells as to where cell proliferation took place.

B. Observation of the ureter
We observed the ureteral epithelium of adult mice (8–12 weeks old) by electron microscopy.

For normal development untreated fetuses were taken out at GD 10.5, 11.5, 12.5, 13.5, 14.5, 15.5, 16.5, or 18.5. In TCDD group, pregnant colony-bred Jcl:ICR mice were treated with TCDD at 40 μg/kg b.w. by gavage at GD 12.5. Fetuses were taken out at GD 13.5, 14.5, 15.5, 16.5, or 18.5.

For electron microscopy, the ureter was fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, stained en bloc in 3% uranyl acetate, dehydrated in graded ethanol series, and embedded in Epon mixture. Ultrathin sections cut horizontally were observed in a transmission electron microscope (Hitachi, H-7100 type). Semithin sections were observed in a light microscope.

3. Results

In the TCDD group at dose levels of 10–80 μg/kg cleft palate or hydroureter was induced dose-dependently when estimated at GD 18.5 (data not shown). At the dose of 40 μg/kg embryolethality was not different from that of controls, and both cleft palate and hydroureter were induced in more than 90% live fetuses.

A. Observation of palatogenesis
In controls by GD 14.25, palatal shelves elevated horizontally and the initial contact took place in the middle region of the shelves. The fusion proceeded in a short period and spread both anteriorly and posteriorly. At GD 14.5 palatal contact seemed almost completed in controls as observed under a dissecting microscope. In histology an epithelial seam, composed of basal cells from each shelf, was seen in the midline at GD 14.5. At GD 15.5 the epithelial seam lost its continuity with epithelial linings facing the oral and nasal cavity and formed epithelial islets.

In the TCDD group, however, both palatal shelves remained in a vertical position at GD 14.25. At GD 14.5 one side or both of the palatal shelves were in a vertical position without horizontal rotation. In addition, the width of palatal shelves in the TCDD group were narrower than that in controls, as estimated in coronal sections.

A–a) Immunohistochemistry of BrdU/ anti-BrdU antibody with serial sections
Uptake of BrdU was observed in some basal cells as well as mesenchymal cells beneath the basal lamina. In addition, a small number of peridermal cells incorporated BrdU as well. Future contact regions (in other words, medial edge areas) could be judged in secondary palatal shelves at GD 13.5 by comparing sections from GD 14.0 or 14.25. At GD 13.5 BrdU-positive epithelial cells were evenly distributed in the shelf. BrdU positive cells in the MEE region decreased in time from GD 13.5 to 14.5 compared with other shelf epithelial cells, that is, future oral and nasal epithelial cells. Neither cells in the midline epithelial seam nor cells in the epithelial islands took up BrdU.

In shelves of the TCDD group, distribution pattern of BrdU positive epithelial cells was almost the same as in controls at GD 13.5, 14.0 and 14.25. In clefted palates, where no midline epithelial seam was formed, MEE cells were not labeled with anti-BrdU antibody at GD 14.25, 14.5, or 14.75. In the TCDD group at GD 15.5, however, MEE cells resumed BrdU incorporation, like other epithelial cells in the shelf facing the oral and nasal cavity.

These results suggest that the pattern of cell proliferation in the TCDD group is similar to that in controls prior to contact.

A–b) Immunohistochemistry of BrdU/ anti-BrdU antibody by CLSM
CLSM confirmed the results obtained with immunohistochemistry of serial sections mentioned above, although the method of BrdU application was different.

B. Observation of the ureter
In the adult ureter, the epithelium was composed of three cell layers; basal, intermediate and superficial cell layers. Basal cells lay on the basal lamina and intermediate cells extend their cytoplasmic processes to the basal lamina, while superficial cells lost contact with the basal lamina. Junctional complexes sealed neighboring superficial cells. In the superficial cell cytoplasm fusiform vesicles were abundant. The apical plasma membrane was made of concave plaques. Immature fusiform vesicles were seen in intermediate cells. The most immature vesicles could be observed in some basal cells. Maturation of the vesicles could be detected from the intermediate to the superficial cells.

In control fetuses, the ureteral bud sprouted from the mesonephric duct close to its entrance to the cloaca at GD 10.5. The epithelium was composed of a single cuboidal epithelial sheet up to GD 14.5. Tight junction was formed at the lateral plasma membrane. With this junctional complex the ureteral lumen was sealed. At GD 16.5 epithelial cells proliferated to become two-cell thick. The basally located cells underwent mitosis. Adult-type superficial cells (Deckzellen) with fusiform vesicles appeared first at GD 18.5, and the epithelium consisted of three-layered cells. Mitotic figures were seen in the basal layer also at GD 18.5. No profiles of physiological cell death were noticed in the ureter in the fetal period.

No apparent ultrastructural difference could be detected in the ureteral epithelium at GD 14.5 between the TCDD group and controls. In the TCDD group, epithelial cells proliferated at GD 16.5 to a greater extent than in controls. In addition, profiles of cell deaths were seen in epithelial cells which faced the ureteral lumen or in basal cells lying on the basal lamina. Disruption of junctional complex between neighboring cells were not conspicuous except in dying cells facing the lumen. At GD 18.5 hydroureter was apparent. Under a dissection microscope, the middle portion of the ureter between the renal pelvis and urinary bladder was narrowed. In this portion cell debris were noticed to fill the ureteral lumen. However, serial sections of the ureter at GD 18.5 in the TCDD group from the narrowed portion down to the vesicoureteral junction failed to show complete obliteration, although cell debris were frequently seen in the lumen. No difference was observed in the vesicoureteral junction between the TCDD group and controls at GD 18.5.

4. Discussion

A. Pathogenesis of cleft palate by TCDD
Results obtained in this study suggest that cell proliferation is suppressed at the MEE region also in the TCDD group during GD 14 (GD 14.0–15.0), although the palatal shelves did not come in contact. It seems inappropriate to ascribe the causative mechanism of cleft palate by TCDD to continuous cell proliferation during contact of palatal shelves.

Together with our previous report[4], it can be said that cell proliferation stops and peridermal cells undergo PCD prior to shelf contact in the MEE region also in the presence of TCDD. It was shown that the elevation of palatal shelves from a vertical to horizontal position was retarded due to TCDD. In addition, palatal shelves in the TCDD group were narrower in width than those in controls. These observations imply that TCDD interferes development and elevation of palatal shelves before initial contact. A question occurs to us; do palatal shelves make contact with each other in the presence of TCDD? As to this question, we have no direct evidence to answer. However, observation of exencephalic fetuses exposed to TCDD might give us another viewpoint as to the pathogenesis of cleft palate. In exencephalic fetuses the palatal shelves are elevated earlier than non-exencephalic fetuses and the posterior portions of shelves are forced to make contact with each other, probably due to the narrowing of the skull base[7]. When exencephalic fetuses were exposed to TCDD, they are resistant to cleft palate induction[8]. These results might imply that palatal closure is completed even in the presence of TCDD, if initial contact does occur.

B. Pathogenesis of hydroureter by TCDD

In the adult ureter it is speculated that the basal cell is the progenitor stem cell, giving rise to daughter cells, and that the daughter cells migrate upward (toward the ureteral lumen) and replace intermediate and superficial cells.

During development, it is clarified that basal cells proliferate as progenitor stem cells and come to form the stratified epithelium.

The observation that cells proliferated excessively at GD 16.5 in the TCDD group suggests that the ureter was narrowed due to the proliferation of ureteral epithelial cells and that dying cells or cell debris shed from the epithelium into the ureteral lumen obliterated the passage of the urine. Cell death in the basal cell might disturb cell turnover of the epithelium in this region, while excessive cell proliferation took place in other regions of the ureter. Some questions remain still unsolved as to whether TCDD induces direct cell death in the epithelium and whether cell death is caused secondarily by the excessive cell proliferation.

5. References

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