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Lower concentrations of receptor for advanced glycation end products and epiregulin in amniotic fluid correlate to chemically induced cleft palate in mice

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ABSTRACT

This study investigated the correlation between differentially expressed proteins in amniotic fluid (AF) and cleft palate induced by all-trans retinoic acid (atRA), and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in mice. Seven proteins were differentially expressed at embryonic day (E) 16.5 in atRA and control groups as revealed by label-based mouse antibody array. Enzyme-linked immunosorbent assay was further used to detect the expression levels of these proteins in AF from E13.5 to E16.5 in atRA, TCDD, and control groups. The cleft palate groups showed lower concentrations of receptor for advanced glycation end products (RAGE) and epiregulin at E16.5. RAGE immunostaining obviously decreased in palatal tissue sections obtained from E14.5 to E16.5 in the cleft palate groups as revealed by immunohistochemistry. These findings indicate that reduced levels of RAGE and epiregulin in AF are correlated to chemically induced cleft palate in mice.

1. Introduction

Cleft palate is a common congenital defect affecting roughly 1 in 2000 live births. The etiology of cleft palate is complex, including multiple genetic and environmental factors (Dixon et al., 2011). Individuals with cleft palate experience problems with feeding, speaking and social integration.

Close similarities of the embryonic processes in the palate between humans and mice have been noted (Meng et al., 2009). In mice, secondary palate starts to develop at embryonic day (E) 12, a period in which palatal primordia emerge from the inner part of the maxillary process. Bilateral palatal shelves subsequently grow vertically on both sides of the tongue at E13. Then, the shelves elevate into a horizontal position above the tongue. At E14-15, the opposing palatal shelves grow toward each other and adhere to form the midline epithelial seam (MES). The MES subsequently disappears, and palatal fusion is completed by E16. Disturbance of this highly regulated process at any stage may result in cleft palate (Gritli-Linde, 2007).

Exposure of mice to a wide variety of chemicals, such as all-trans retinoic acid (atRA) (Abbott et al., 1989), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Yoon et al., 2000), or hydrocortisone (Erickson et al., 2005), causes cleft palate. atRA is a metabolic product of vitamin A, whereas TCDD is a highly toxic and stable environmental contaminant (Abbott and Birnbaum, 1989). The mouse cleft palate model induced by atRA or TCDD is well established and widely used in research on the defect.

Amniotic fluid (AF) is a clear liquid protecting the fetus. Protein profiles in AF vary as gestation progresses, reflecting both the physiological and pathological changes affecting the fetus and the mother (Orczyk-Pawilowicz et al., 2005). Protein analysis of AF is the ideal first step in clarifying the variations related to pathological conditions of a fetus. For instance, higher levels of alpha-fetoprotein in AF are correlated to open neural tube defects (Loft et al., 1993), and reduced concentrations of some B-vitamins in the AF are associated with cleft lip and/or palate appearance in the offspring of A/WySn mice (Scheller et al., 2013). To date, few studies have conducted protein analysis of AF associated with cleft palate.

In this study, we used the label-based mouse antibody array to compare the protein levels of AF from cleft palate embryos induced by atRA at E16.5 with those from healthy control. Differential expression levels of these proteins in AF and in palatal

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tissue with palatogenesis were determined through enzyme-linked immunosorbent assay (ELISA) and immunohistochemical staining (IHC) to verify the proteins correlated to chemically induced cleft palate.

2. Materials and methods

2.1. Chemicals and reagents

atRA (CAS no. 302-79-4; MW, 300.44; purity ≥ 98%) was purchased from Sigma (St. Louis, MO, USA). atRA powder was dissolved and stored in dimethyl sulfoxide. All works involving atRA were conducted under dim yellow light to prevent photo-oxidation. TCDD (CAS no. 1746-01-6; MW, 321.9; purity, 99.8%) was obtained from AccuStandard (NH, USA). TCDD powder was dissolved and stored in toluene. Dose formulations for treatment were prepared at E10.5 by mixing the atRA solution or TCDD solution in corn oil vehicle. Hematoxylin and eosin were purchased from Wuhan Chemical Reagent Factory (Wuhan, China). Label-based mouse antibody arrays were obtained from RayBiotech, Inc. (Norcross, GA, USA). All ELISA kits were obtained from R&D Systems, Inc. (Minneapolis, USA). Rabbit SP kit was purchased from Zhongshan Golden Bridge Biotech (Beijing, China). The rabbit polyclonal RAGE antibody was obtained from Proteintech Group, Inc. (Wuhan, China).

2.2. Animals and exposure

Wild-type 8-week-old C57BL/6J mice were purchased from Experimental Animal Research Center of Hubei Province (Wuhan, China) and housed under specific-pathogen-free conditions. Mice were mated for 12 h, and the presence of a vaginal plug was designated as E0.5. Ninety pregnant mice were randomly divided into three groups at E10.5, given a single dose of atRA (100 mg/kg) or TCDD (40 μg/kg) dissolved in corn oil by oral gavage, or an equal amount of corn oil in control group. Fetuses and AF obtained at E13.5 to E16.5 were harvested (E13.5–E15.5: 5 mice per stage and group, E16.5: 15 mice per group). The experimental protocols were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of Wuhan University.

2.3. AF sample (AFS) collection and preparation

The AFS of each fetal sac was aspirated and preserved in 1.5 ml centrifuge tubes. To obtain a sufficient amount of protein for several rounds of experiments and to reduce the individual difference, we pooled AFS from each pregnant mouse into a tube, obtaining a protein mixture. AFS contaminated by blood or AFS from dead fetuses or fetuses with malformations were excluded. All fetuses were examined for the presence of cleft palate by using a stereomicroscope. The number of embryos and volume of AF at E16.5 in the three groups were recorded. Fresh AFS were immediately centrifuged at 5000*g* at 4 °C for 5 min to separate cell debris. The samples were subsequently stored at −80 °C for further experiments.

2.4. Hematoxylin and eosin (HE) staining

Embryo heads obtained from three groups at E16.5 were dissected for HE staining (n = 5 litters per group). They were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned (5 μm), and stained with hematoxylin and eosin for microscopic observation.

2.5. Mouse antibody array

RayBio® Biotin label-based mouse antibody array 1 (AAH-BLG-1) was used to compare control and atRA groups in terms of protein expression levels in AFS from E16.5 when palatal fusion is completed (n = 5 litters per group). This array simultaneously detects 308 mouse target proteins, including cytokines, chemokines, adipokine, growth factors, and soluble receptors. The procedure for protein expression screening using this antibody array was described extensively by Ding et al. (2014).

2.6. ELISA

Concentrations of receptor for advanced glycation end products (RAGE), epiregulin, leukemia inhibitory factor (LIF), interleukin-10 (IL-10), interleukin-12 p40 (IL12-p40), interleukin-12 p70 (IL12-p70), interferon-beta (IFN-β), and interferon-gamma (IFN-γ) in AF were measured by ELISA kits. AFS were collected from three groups at E13.5 to E16.5 (E13.5–E15.5: n = 5 litters per stage and group, E16.5: n = 10 litters per group). Each sample was measured in duplicate according to the manufacturer's protocol.

2.7. Immunohistochemistry

Embryo heads obtained from E13.5 to E16.5 from the three groups were dissected and fixed in 4% paraformaldehyde overnight at 4 °C for IHC (n = 5 litters per stage and group). The 5-μm paraffin sections were deparaffinized, rehydrated, and subjected to antigen retrieval by using a high-pressure method. A mixture of 30% H_2O_2 and methanol (1/9, v/v) was used to inhibit endogenous peroxidase activity. The sections were incubated overnight with the rabbit polyclonal RAGE antibody (1:50 dilution) at 4 °C and then detected using a rabbit SP kit. The sections were subsequently counterstained with hematoxylin. A scoring method combining the degree of staining intensity with the percentage of stained cells (extensiveness) was assigned to each case. Two personnel were trained to evaluate the staining intensity and extensiveness according to the criterion presented by Katz prior to the conduct of formal examination (Katz et al., 2009). Staining intensity was rated as follows: no visible staining = 0, faint staining = 1+, moderate staining = 2+, and strong staining = 3+. Extensiveness was estimated semi-quantitatively as 0%, <10%, 10%–25%, 25%–50%, 50%–75%, 75%–90%, and >90% of positively stained cells in each high-power field. Kappa scores of the two personnel were greater than 0.75 after the training. They were blind to the palatal status of the embryos they scored.

2.8. Statistical analyses

Kolmogorov–Smirnov and Shapiro–Wilk tests were used to determine whether data were normally distributed. All quantitative data were presented as mean ± standard deviation, and data were analyzed using one-way or two-way ANOVA, or Kruskal–Wallis and post-hoc Mann–Whitney *U* tests at a significance level of $p < 0.05$.

3. Results

3.1. atRA or TCDD administration at E10.5 induces cleft palate

The morphological outcomes in terms of palatal development of E16.5 fetuses treated with atRA and TCDD were examined under a stereomicroscope. An oral dose of atRA (100 mg/kg) given to pregnant mice at E10.5 induced cleft palate in all of the fetuses (15 mice/117 fetuses), and the cleft palate was not accompanied by other obvious craniofacial defects (Fig. 1A). Pregnant mice given