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Perturbation of Fgf10 signal pathway in mouse embryonic palate by dexamethasone and vitamin B_{12} in vivo

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Abstract

Background/Purpose: The Fgf10 signaling pathway plays an important role in early stages of mouse embryonic palatal development, which is associated with cell proliferation and differentiation. The objective of this study was to assess whether dexamethasone and vitamin B_{12} affected the Fgf10 signal pathway of mouse embryonic palate.

Materials and Methods: Immunohistochemical studies were performed for expression of Fgf10, Fgfr2b, and sonic hedgehog and for cell proliferation and apoptosis of mouse embryonic palate.

Results: The expression of Fgf10, Fgfr2b, and sonic hedgehog was changed in mouse embryonic palate after dexamethasone and vitamin B_{12} treatment, resulting in reduced and restored proliferation of mesenchymal cells.

Conclusions: Dexamethasone and vitamin B_{12} affected the Fgf10 signaling pathway and cell proliferation of mouse embryonic palate. Cell apoptosis was not altered after dexamethasone and vitamin B_{12} exposure.

Nonsyndromic cleft palate is a frequently encountered congenital malformation in humans, which may result from failure of palatal growth and elevation as well as fusion. Environmental factors, such as glucocorticoids (GC), are important causes for isolated cleft palate (CP) in addition to a genetic predisposition. It has been reported that the mesenchymal cell proliferation was decreased, and medial edge epithelium (MEE) cells differentiate into the stratified squamous epithelial cells when rat embryonic palatal shelves were exposed to GC and resulted in CP [1]. Study of molecular biology showed that GC affected expression of growth factor in MEE cells of mouse palatal shelves [2]. However, dexamethasone (DEX)-induced growth inhibition of mouse embryonic palatal mesenchymal (MEPM) cells was not mediated by transforming growth factor β (TGFβ) [3]. These results suggested that GC-induced CP might inhibit MEPM cell proliferation rather than altered MEE cell differentiation. The molecular mechanism of DEX-induced growth inhibition of MEPM remains largely unknown.

Vitamin B acts as a favorable factor in decreasing the incidence of CP [4]. A decreased level of vitamin B_{12} (VitB_{12}) in maternal blood may act as a risk factor for malformations of the embryonic palate [5]. The incidence of DEX-induced mouse CP was decreased after VitB_{12} exposure [6]. However,

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$VitB_{12}$ had no direct effects on the gene expression of epidermal growth factor, TGFα, and TGFβ [7].

A previous study indicated that the fibroblast growth factor (FGF) signaling pathway may contribute to 3% to 5% of nonsyndromic cleft lip with or without CP and may be considered in the clinical management of cleft lip with or without cleft palate (CL/P) [8]. Fgf10 is secreted by palatal mesenchymal cell and binds to its specific receptor FGF-R2-IIIb (Fgfr2b) that is expressed on proliferated MEE and induces sonic hedgehog (Shh) expression by the overlying epithelium. The Shh induces bone morphogenetic protein 2 (Bmp2), which is expressed on the mesenchyme and activates cell proliferation [9]. Recent studies showed that a mutant of the Fgfr2b receptor in mouse models resulted in decrease proliferation in the palatal epithelium and mesenchyme, thus causing a CP phenotype [9].

The present study was designed to test the hypothesis that GC-affected palate development may be contained by $VitB_{12}$ through change in the balance between apoptosis and proliferation of MEPM cells. At the same time, the expression of Fgf10/Fgfr2b/Shh signals was altered.

1. Materials and methods

1.1. Animals

Mature male and female C57BL6/J mice were housed at a temperature of 20°C ± 5°C with a 12-hour light/dark cycle. Animals were maintained in mouse cages in pathogen-free conditions with food and water ad libitum. Matings were accomplished by placing 1 male and 2 females in a cage overnight. A vaginal plug was found the following morning, which was considered as evidence of mating gestation day 0 (GD 0).

1.2. Treatment and sample collection

Dams were injected intraperitoneally with normal saline 0.1 mL, DEX 6 mg/kg, DEX 6 mg/kg, and $VitB_{12}$10 mg/kg every day at GD 10 to 12, respectively. Pregnant mice were killed at GD 12.5, 13.5, and 17.5. Three dam-allocated fetuses were analyzed per dam at GD 12.5 and 13.5. Eight dams were allocated, and all fetuses were analyzed at GD 17.5. At GD 12.5 and 13.5, embryonic heads per time-point were fixed in 4% paraformaldehyde in phosphate-buffered saline solution overnight at 4°C and embedded in paraffin for histological and immunohistochemical investigation. At GD 17.5, embryonic heads were observed for the number of CP by the naked eye.

1.3. Immunohistochemical stain

For immunohistochemistry, the palatal shelves at GD 12.5 to 13.5 were fixed with 4% paraformaldehyde and embedded in paraffin. Sections were cut (7 μm) and stained with anti-fgf10, fgfr2b, and Shh (Santa Cruz, Calif.), respectively, according to a standard procedure [10]. Biotin-labeled secondary antibody was from Santa Cruz.

1.4. 5-Bromodeoxyuridine assay

For 5-bromodeoxyuridine (BrdU) labeling in vivo, timed pregnant mice were injected intraperitoneally with BrdU labeling reagent (50 mg/kg body weight). Mice were killed, and embryos were harvested 2 hours after injection. Samples were performed with the BrdU labeling and Detection Kit (Beijing Biosynthesis Biotechnology Co, China) according to the manufacturer's protocol. Embryonic specimens were fixed, dehydrated, embedded in paraffin wax, and sectioned at 7 μm. The primary antibody incubations were carried out at 37°C for 2 hours and 4°C overnight, and second antibody incubations were carried out at 37°C for 30 minutes in a humidified chamber. Hematoxylin counterstain visualizes the sites of BrdU incorporation. BrdU-positive cells and all cells in the palatal mesenchyme within the fixed area were counted (100 μm^2). Three continuous sections from the palatal shelves before first molar in each palatal shelf from each individual embryo were counted. Cell proliferation in palatal mesenchyme was analyzed for the ratio of positive to total number of cells.

1.5. TUNEL assay

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed using ApopTag Plus Fluorescein In Situ Apoptosis Detection Kit (Oncor, Gaithersburg, Md) following the manufacturer's instructions. Briefly, tissues were fixed in 4% paraformaldehyde (PFA) (in phosphate-buffered saline) and then dehydrated through xylene, decreasing graded ethanol series, and processed for sectioning. Following rehydration steps, the 7-μm sections were treated with proteinase K (in 10 mmol/L Tris-HCl, pH 8.0) at a concentration of 20 μg/mL for 15 minutes at room temperature. The samples were incubated with the equilibration buffer for 10 seconds and then immediately were incubated with working strength TdT enzyme in a humidified chamber at 37°C for 1 hour. The samples were then treated with working strength stop buffer for 10 minutes at room temperature and with working strength anti-digoxigenin conjugate to the slide, incubated in a humidified chamber for 30 minutes at room temperature to avoid exposure to light and viewed by fluorescence microcopy.

Table 1 The number of fusion and CP in each group

	Fusion	Cleft	Total
Control group	45	2	47
DEX-treated group	20	28 *	48
DEX+$VitB_{12}$-treated group	36	8	44

* $P < .05$, significantly different from the control group and $VitB_{12}$-treated group.