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Lithium inhibits palatal fusion and osteogenic differentiation in palatal shelves *in vitro*

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ABSTRACT

Objective: Glycogen synthase kinase-3β (Gsk-3β)/β-catenin signaling regulates development of the secondary palate. It has been unclear about the effects of Gsk-3β/β-catenin signaling on palatal fusion and osteogenic differentiation in palatal shelves.

Design: In this study, palatal shelves from mouse embryonic day 13 (E13) were cultured *in vitro* with or without lithium chloride (LiCl). Palatal fusion was evaluated by haematoxylin-eosin staining. The expression of osteogenic markers in palatal shelves was measured by quantitative PCR, and immunohistochemical staining. Cell proliferation and apoptosis were examined by Ki-67 immunohistochemical and TUNEL staining, respectively. Gsk-3β expression was evaluated by quantitative PCR and Western blotting. β-catenin protein expression was evaluated by Western blotting.

Results: After the treatment with 10 mM LiCl, palatal shelves failed to fuse, and the mRNA and protein levels of osteogenic markers were reduced compared with controls. The number of Ki67-positive cell in the palatal osteoid was significantly higher in the LiCl group than in the controls. The apoptotic cells in the midline epithelial seam were reduced by LiCl. Gsk-3β mRNA and protein expression levels decreased and β-catenin protein expression levels increased by treatment of LiCl.

Conclusion: Our findings show that LiCl-mediated GSK3β inhibition prevents palatal fusion and osteogenic differentiation in palatal shelves by increased β-catenin signaling. It indicated that overactivation of canonical Wnt signaling might impair the fusion of the secondary palate.

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1. Introduction

Cleft palate is a common congenital defect, affecting roughly one in 2000 newborn babies worldwide. The etiology of clefting includes multiple environmental and genetic factors[1,2]. In mice, the development of the secondary palate starts from the palatal primordia at the lateral edges of the maxillary process at embryonic day 12 (E12)[3]. The bilateral palatal shelves subsequently grow down vertically along the two sides of the tongue. Then, the palatal shelves elevate above the dorsum of the tongue. At E14–15, the bilateral palatal shelves grow toward each other and adhere to form the midline epithelial seam (MES). The MES then disappears and palatal fusion is completed by E16. At this time, mesenchymal condensation also occurs, followed by osteogenic differentiation of the palatal mesenchyme, which eventually leads to the formation of the palatal bone. Disturbance of this highly regulated process may result in a failure of fusion of the palatal shelves and, hence, in a cleft palate[4].

Glycogen synthase kinase-3β (Gsk-3β), a serine/threonine protein kinase, is crucial in Wnt/β-catenin signaling. When Wnts bind to the cell surface receptors of the Frizzled family and the lipoprotein receptor-related protein (LRP) 5/6, Gsk-3β activity is inhibited, resulting in accumulation of cytoplasmic β-catenin. Then, β-catenin translocates into the nucleus and regulates downstream signal transduction pathways[5]. The inactivation of Gsk-3β in mice causes cleft palate, possibly through Wnt/β-catenin signaling [6,7]. However, Wnt/β-catenin signaling is also required for palatal fusion[8]. Lithium chloride (LiCl) is one of the most effective drugs for the treatment of bipolar (manic-depressive) disorder[9]. It activates Wnt/β-catenin signaling by inhibiting GSK-3β[10]. The administration of lithium results in several congenital defects in the fetus, including cleft palate[11,12]. In addition, LiCl regulates the expression of osteogenic factors such as alkaline phosphase and osteocalcin, and bone formation[13–18]. However, it is still unknown about the effects of increased β-catenin on palatal fusion and osteogenic differentiation in palatal shelves. Thus, the purpose of our current study was to investigate the effects of LiCl on palatal fusion and osteogenic differentiation in cultured palatal shelves.

2. Materials and methods

2.1. Tissue culture

Wild-type CD1 mice (Charles River) were mated for 4 h, and the presence of a vaginal plug was designated as embryonic day (E) 0. Pregnant mice were killed at embryonic stage E13. Palatal shelves were cultured according to the method of Brunet et al. (1995)[19]. Briefly, 3–5 pairs of palatal shelves were placed on 0.8 μm Millipore filters on a metal grid in a 6-well plate with their medial edge epithelia (MEE) in close apposition. The palatal shelves were cultured in 4 ml Dulbecco's Minimal Essential Medium/Ham's F12 Growth Medium (DMEM/F12) 1:1 (GIBCO-BRL, Breda, The Netherlands), supplemented with 1% L-glutamine, 1% ascorbate, and 1% penicillin/streptomycin in 95% air and 5% CO_2 at 37 °C. Tissues were treated with 10 mM lithium chloride (LiCl) (Sigma-Aldrich, Zwijndrecht, the Netherlands) or phosphate buffered saline (PBS), and collected after 72 h. In preliminary experiments, palatal shelves were cultured with various concentrations of LiCl and then stained with haematoxylin-eosin. While high doses of LiCl (≥20 mM) were toxic (data not shown), lower doses were well-tolerated. The experiments were approved by the Board for Animal Experiments of the Radboud University Nijmegen, The Netherlands.

2.2. Histochemical and immunohistochemical staining

6–12 paired palatal shelves were used for each group in these experiments. Cultured palatal shelves were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned (5 μm). One frontal section in every ten sections from the palatal shelves was stained with haematoxylin-eosin for fusion rate survey. Three sections were taken from the anterior, middle, and posterior parts of the palatal shelves, respectively. Totally, nine sections from each pair of palatal shelves were mounted on SuperFrost/Plus slides for each experiment (Menzel-Gläser, Braunschweig, Germany).

For alkaline phophatase staining, deparaffinated and rehydrated sections were incubated in 0.1 M Tris/HCl buffer (pH 9.5) containing 50 mM $MgCl_2$ and 100 mM NaCl for 10 min, and then put in the same buffer containing 0.5% nitro blue tetrazolium chloride and 0.375% 5-Bromo-4-chloro-3-indolyl phosphate toluidine salt (Roche Diagnostics, Mannheim, Germany) at 37 °C for 20 min. The sections were rinsed in water and counter-stained with 0.1% methyl green.

For immunohistochemistry, the deparaffinated sections were treated with citrate buffer (pH 6.0) in a microwave oven for 10 min, then with 1% trypsin (Difco Laboratories, Detroit, MI, USA) at 37 °C for 5 min, and finally with a mixture of 30% H_2O_2 and methanol (1/9, v/v) to inhibit endogenous peroxidase activity. The sections were then rinsed in PBS and pre-incubated in 10% normal donkey serum (NDS, Biomeda, Foster City, USA) for 15 min. The sections were incubated with the first antibody in 2% NDS in PBS at 4 °C overnight (osteocalcin, 1:400, Santa Cruz Biotechnology, CA, USA; Ki67, 1:200, Euro-Diagnostica, Arnhem, The Netherlands). The sections were incubated with a biotinylated donkey secondary antibody (Jackson ImmunoResearch, Westgrove, PA, USA). The sections were then incubated with ABC-peroxidase (Vector Laboratories, Burlingham, CA, USA), which was visualized with DAB. The staining was enhanced with 0.5% $CuSO_4$ in 0.9% NaCl. Sections without the primary antibodies were used as a negative control and were always blank. Ki67 positive cells were counted in the area of almost 0.02 mm^2 in the osteoid and the rest of palatal mesenchyme by use of an ocular grid, respectively.

For the TUNEL assay, palatal shelves were cultured for two days. Then, tissues were collected and TUNEL assays were performed to detect apoptotic cells according to the manufacturer's protocol (Roche, Indianapolis, IN, USA). TUNEL-positive cells were counted on the MES of palatal shelf sections from control and LiCl groups, respectively.

2.3. Real-time quantitative polymerase chain reaction

6 Paired palatal shelves were used for each group in this experiment. Three pools of 2–3 paired palatal shelves were