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ClC chloride channels in tooth germ and odontoblast-like MDPC-23 cells

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ABSTRACT

Objective: To detect expression of ClC chloride channel mRNA in tooth germ and odontoblasts, and explore the affect of chloride channel function on cell proliferation and cell cycle.
Design: We extracted total RNA of tooth germ from newborn C57BL mice and mouse odontoblast-like cells (MDPC-23), then detected mRNA expression of chloride channel genes Clcn1–7 with RT-PCR. We used chloride channel blocker 5-nitro-2-(3- phenylpropylamino)-benzoic acid (NPPB) to interfere with chloride channel function of MDPC-23 cells. Cell proliferation rate and cell cycle were detected with MTT assay and flow cytometry, respectively. Student's *t*-test was used to determine statistical significance between control and treatment groups.
Results: The mRNA of Clcn1–7 chloride channel genes was expressed in tooth germ of newborn mice. Clcn3, Clcn5 and Clcn7 mRNAs were expressed in MDPC-23 cells. NPPB slowed down the proliferation rate of MDPC-23 cells from day 2 to day 4 ($P < 0.01$), and also changed the proportion of cell cycle phase. Comparing to the control, the proportion of G2/M phase cells reduced from $3.93 \pm 2.62\%$ to $0.54 \pm 0.25\%$ ($P < 0.05$). The ratio of G1/G2 increased from 1.86 ± 0.01 to 1.95 ± 0.02 ($P < 0.05$).
Conclusions: There is abundant chloride channel gene expression in tooth germ. Some of these chloride channels may regulate tooth development through effects on cell proliferation and cell cycle signal pathway.

1. Introduction

Chloride channels are ubiquitously expressed in almost all eukaryotic cells, and play important roles during physiological and biological processes, such as neuronal and muscle excitability, cell volume regulation, transepithelial ion transport, calcium modulation, cellular pH and cell proliferation.[1] Chloride channels are transmembrane proteins that form pores which allow the passive transport of chloride ions (Cl^-).[2] In the past decade, remarkable progress has been made in the understanding of human diseases involving chloride channels. Some genetic diseases in muscle, kidney, bone and brain are due to mutations in chloride channel genes which lead to a loss of channel function.[3] Chloride channels are typically classified into four groups: voltage-dependent (CIC), cystic fibrosis transmembrane conductance regulator (CFTR), calcium-activated (CaCC), and glycine- or γ-aminobutyric acid (GABA)-activated.[3,4]

In mammals, the CIC chloride channel family is comprised of nine voltage-gated chloride channels, known as Clcn1–7, Ka, and Kb. Based on sequence homology, these genes are divided into three subgroups. The physiological and

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pathophysiological roles of several ClC channels are also illuminated by a variety human inherited diseases caused by mutations in ClC genes.[5,6] One important example is ClC-5, derived from the Clcn5 gene. Mutations of Clcn5 causes Dent's disease, which is characterized by nephrolithiasis and hypercalciuria.[6] Clcn5 gene knockout mice show symptoms similar to Dent's disease. Another interesting feature of this animal model is that Clcn5 knockout mice show deformities of the dorsal spine and backward growth of the teeth.[7]

Interestingly, there are some chloride channels in odontoblasts, the cells responsible for the formation of dentine that constitute the bulk of teeth.[8] The first physiological study about chloride channel in tooth was reported in 1998. Guo and Davidson found that there are some chloride-selective channels in freshly isolated odontoblasts, the cells which produce dentin. These channels are weakly permeable to both acetate and aspartate, and had a conductance of 391 ± 64 pS and 24 ± 3 pS.[9] Another report asserts that odontoblasts have chloride channels which may allow inorganic sulphate into the cell.[10]

In contrast to the extensive knowledge of chloride channels in other organs, little is known about chloride channel function in tooth formation, calcification and eruption. One report showed there were two separate anion channels in the odontoblast membrane which were predominantly chloride-selective.[9] Results from chloride channel gene knockout mice suggest that CFTR and ClC-5 chloride channels may also regulate tooth development.[7,11] CFTR knockout mice have enamel with crystallite defects, retained protein, and hypomineralization, which suggests that CFTR is involved in enamel formation and mineralization.[11,12]

The aim of the present study was to characterize chloride channel function in tooth and odontoblasts. We detected the expressions of ClC family members (Clcn1–7) in tooth germ as well as the odontoblast-like cell line, MDPC-23. We also applied the well characterized chloride channel blocker, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), to MDPC-23 cells and observed effects on cell proliferation and cell cycle.

2. Materials and methods

2.1. Cell culture

The MDPC-23 odontoblast-like cell line was derived from 18–19-day-old CD-1 foetal mouse molar dental papillae (gift from Prof. C.T. Hanks and Dr. Jacques E. Nor, University of Michigan). This cell line retains several unique features and characteristics of odontoblasts. [8] MDPC-23 cells were cultured in α-MEM Medium (Gibco, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 2 mmol/L L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin, and in a humidified atmosphere containing 5% CO_2, 95% CO_2.

In the blocking experiments, NPPB (Sigma, St Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) to a 100 mM stock solution and diluted with α-MEM Medium. The working concentration of NPPB was 50 μM, and the same volume of dimethyl sulfoxide was used as control.

2.2. Reverse-transcriptase-polymerase chain reaction (RT-PCR) analysis

The expression of ClC channels in tooth germ and MDPC-23 cells was evaluated by RT-PCR. Total RNA of tooth germs (containing both incisor germs and molar germs) from newborn C57BL mice and MDPC-23 cells (70–80% confluent) was isolated using TRIzol® Reagent (Life Technologies, Gibco BRL), according to the manufacturer's protocol. Concentration of RNA was determined by spectrophotometer (Bio-Rad, USA) at 280/260 nm. M-MLV Reverse Transcriptase cDNA Synthesis Kit (Invitrogen, USA) was used to synthesize cDNA by the manufacturer's instruction. The primers for detecting ClC channels are shown in Table 1. PCR reaction was performed in a total volume of 50 μL, with Platinum Taq DNA polymerase (Invitrogen) or rTaq DNA polymerase system (TaKaRa, Japan). The final primer concentration was 0.2–0.5 μM. PCR was performed in a PCR machine (GeneAmp PCR System 2400, PerkinElmer, USA) under general PCR condition parameters, and annealing temperatures were maintained between 55 °C and 60 °C. The PCR product was analysed by electrophoresis on a 1.0–1.5% agarose gel.

2.3. MTT assays

MDPC-23 cells were seeded at a density of 400 cells/well in 96-well plates (Corning-Costar Corp., USA) containing 200 μL of culture medium, and allowed to attach overnight. After 24 h, the medium was removed and replaced with the medium containing 50 μM NPPB or control medium. The cells were treated with NPPB for 1 day, 2 days, 3 days, 4 days, or 5 days. At the end of culture, 20 μL of MTT (5 mg/mL, Sigma, St Louis, MO, USA) was added to each well, and plates were placed at 37 °C for 4 h. Then the medium in each well was replaced with 150 μL DMSO, and the plates were shaken to dissolve the

Table 1 – Primer sequences for detecting Clcn genes and expected PCR products

Gene name	Forward primer (5′–3′)	Reverse primer (5′–3′)	PCR product size (bp)
Clcn1	CGAAAGCACAAGTGTCTCAG	GTCAAAGCTGCTGCTCCAAT	470
Clcn2	CTCAGCAAGTTCCTCTCCCT	GCCACTAGCAATGCCAATGAC	342
Clcn3	CCAAGACCCCGCTTCAATAA	CGAGTCCCGCAGATTAAAGA	112
Clcn4	GCGTCTCATCGGGTTTGC	TTGCTCACAATGCCCTCTTG	84
Clcn5	TGTCGGAAGCGTAAAACCAC	TCCGAGTCACACCGCCTAAG	520
Clcn6	GTCTTGATAGAGCCAGTGGCA	GAAGAGCACTTTCCACGTGAG	405
Clcn7	GAAAGGAAGGGCCAATGATC	CAGGAACTGATTCCAGAAGG	236