Live cell imaging of intracellular calcium responses in the rat dental epithelial cells

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Health Sciences University of Hokkaido

Graduate School of Dentistry

Kaori Murata

Introduction

The tooth germ is the aggregates of epithelial cells and mesenchymal cells. Epithelial-mesenchymal interactions regulate the cell arrangement, migration, growth, morphogenesis, proliferation, differentiation and cell death. It is important that the cell populations cooperate in ectodermal organ such as teeth. Ca^{2+} signals are crucial second messengers in a wide range of biological processes, and modulate various cellular functions including gene expression, proliferation, and differentiation.

The store-operated Ca^{2+} entry (SOCE) is main influx mechanism in many cells. Recent studies have shown that Stim1 and Orai1 are essential molecules in controlling SOCE, and that the deficiency of these molecules causes amelogenesis imperfecta type 3. The change of Ca^{2+} density (Ca^{2+} responses) may control the gene expression and the cell migration in amelogenesis. Rat dental epithelial cell line SF2 is a preameloblast cell line which secretes enamel matrix protein after the differentiation into the ameloblast. It is also known that the active form of vitamin D (1,25-dihydroxyvitamin D3; VD3) regulates growth and differentiation of dental epithelial cells.

To examine the roles of SOCE on the cell differentiation and migration, Ca^{2+} responses were visualized in cultured SF2 cells using gene encorded calcium indicators, G-GECO and YC-Nano50.

Material and method

Materials and media

Rat dental epithelial cells (SF2) were gifted from Dr. Fukumoto. CMV-G-GECO1.1 was a gift of Robert Campbell (Addgene plasmid #32445).

The active form of vitamin D (1α, 25-dihydoroxyvitamin D3: VD3, Sigma, MO, U.S.A) was diluted to 100 nM by dimethyl sulfoxide (DMSO, Wako Pure Chemical Industries, Osaka, Japan).

Cell culture and transfection

SF2 cells were cultured in Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (Ham) (DMEM/F12, Gibco, MA U.S.A) supplemented with 10% fetal bovine serum at 37℃ and 5% CO_2. For imaging, cells were cultured in a glass-bottom dish (Iwaki, Shizuoka, Japan). Transient transfections with the G-GECO that is the Ca^{2+} sensor protein were performed using an adenoviral vector, and incubated for 15-20 hours. SF2 cells were cultured in D-MEM/Ham's F12 (Wako Pure Chemical Industries) which didn't include phenol red during

the live cell imaging.

Cell proliferation assay

SF2 cells were plated $1x10^5$ cells/ml and were cultured in DMEM/F12 supplemented with or without VD3, and the number of cells was counted.

Alizarin red S staining and ALP assay

SF2 cells were plated at $1x10^5$ cells/ml and were cultured in osteogenic medium (minimum essential medium supplemented with 50 mg/ml ascorbic acid and 10 mM β-glycerophosphate) for 10 days.

The cells were fixed with 70% ethanol and stained with Alizarin red S. Alkaline phosphatase activity was measured spectrophotometrically using *p*-nitrophenol as a colorimetric substrate.

Immunostaining

The primary antibodies, 1:200 anti-connexin 43 (Invitorogen , CA , U.S.A), 1:200 anti-ameloblastin (Santa-Cruz, Texas, U.S.A) were utilized. The secondary antibody, 1:200 Alexa fluor 488 (Invitrogen) was used. Immunohistochemical analyses were performed using antibodies listed bellow, and the fluorescence images were captured with confocal laser scanning microscopy.

Live cell Ca^{2+} imaging with G-GECO

For monitoring $[Ca^{2+}]i$, cells were attached to a small recording chamber that consisted of a 10 mm ring and ϕ12 mm glass coverslips. G-GECO-expressing cells were washed with culture medium which does not contain phenol red, prior to Ca^{2+} measurements. For monitoring G-GECO fluorescence, cells were excited at 488 nm, and selected the G-GECO fluorescence using 515 nm band-pass filter (half width 30 nm). The fluorescence images were captured with confocal laser scanning microscopy during the incubated at 37°C in 5% CO_2-Air at 2 minute intervals and the transmitted images were acquired. Cells were maintained under cell culture conditions during imaging using a stage chamber and a stage-and-objective lens heating system (Tokai Hit, INU-NI-F1). The confocal laser scanning microscopy equipped with a Nikon Fluor ×40 oil immersion objective (NA=1.30). For elucidating the mechanism of the Ca^{2+} responses, cells were treated with 1mM $LaCl_3$ (Wako Pure Chemical Industries), 50 μM $CdCl_2$ (Wako Pure Chemical Industries), nonselective plasma membrane Ca^{2+} channel inhibitor, and co-treatment 10 μM thapsigargin (ThG, Wako Pure Chemical Industries), non-competitive inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor and 1 mM $LaCl_3$.

Live cell Ca^{2+} imaging with YC-Nano50

The Ca^{2+} sensor protein, YC-Nano50, was expressed using an adenoviral vector, and the fluorescence images were captured using dual-wavelength ratio imaging system consisting of an EM-CCD camera and W-View optics coupled to a Nikon TE2000 inverted fluorescence microscope or using total internal reflection fluorescence (TIRF) microscopy during the incubated at 37°C in 5% CO_2-Air. Data were analyzed with AQUACOSMOS 2.6 software.

Cell migration

Quantitative analysis of the cell migration was used 96 well plate (*Oris™ Cell Migration Assay*) in the presence or absence of Serum (10% FBS), EGF (100 ng/ml), CXCL12 (50 ng/ml) or $LaCl_3$ (1 mM).

Statistical analysis

All results shown are means ± s.e.m. for three or more independent experiments.

P-values <0.05 represent statistically significant differences.

Results

VD3 reduced the proliferation of SF2 cells and enhanced the expressions of AMBN and CX43. The expression of AMBN was observed at 24 hours after the incubation with VD3, and the number of AMBN-expressing cells increased at 72 hours. The distributions of CX43 in VD3-treated cells showed a punctuated pattern, whereas the control cells were showed more dispersed distribution. Alizarin red S staining and ALP assay indicated promotion of the calcium nodule formation and increased ALP activity by VD3.

Ca^{2+} responses in SF2 cells were examined using live cell imaging techniques. G-GECO-expressing cells showed the intermittent rises in $[Ca^{2+}]_i$ in the cell culture conditions at 37℃ in the absence of external stimuli. The frequency of these spontaneous rises in rises in $[Ca^{2+}]_i$ in SF2 cells were increased in the presence of VD3. The non-selective Ca^{2+} entry inhibitor, $CdCl_2$, and SOCE inhibitor, $LaCl_3$, failed to block the intermittent rises in $[Ca^{2+}]$i, while the combination of ThG and $LaCl_3$ blocked these Ca^{2+} responses almost entirely.

The cell migration of SF2 cells was potentiated in the presence of serum, or EGF plus CXCL12 (without serum). $LaCl_3$ inhibited the serum-dependent cell migration of SF2 cells almost completely, but had no effect on the EGF plus CXCL12-dependent cell migration. Experiments with YC-Nano50 showed that $[Ca^{2+}]_i$ in SF2 cells in the presence of serum and the presence of EGF plus CXCL12 (without serum) were reduced by $LaCl_3$.

Discussion

The present study demonstrates that VD3 reduced the proliferation of SF2 cells, and enhanced the expressions of AMBN and CX43. In addition, VD3 promoted the formation of calcium nodules, and increased ALP activity. These results indicate that VD3 induced differentiation of SF2 cells.

It is also found that VD3 enhanced Ca^{2+} responses of SF2 cells in cell culture conditions. These spontaneous Ca^{2+} responses were not blocked by SOCE inhibitor, $LaCl_3$. On the other hand, experiments with high sensitivity Ca^{2+} indicator, YC-Nano50, indicated the decrease in resting $[Ca^{2+}]i$ by $LaCl_3$. It is also clarified crucial roles of SOCE on the serum-dependent cell migration of SF2 cells. This finding raises a possibility that deficiency of SOCE may cause problems on the enamel organ morphogenesis. For elucidating the mechanism of amelogenesis imperfecta by the deficiency of SOCE, it is important to clarify the molecular mechanisms for the Ca^{2+}-dependent cell migration and to identify genes (molecules) in which their expressions are regulated in a Ca^{2+}-dependent manner.

Conclusion

Vitamin D3 induced differentiation and increased the frequency of spontaneous Ca^{2+} responses in rat dental epithelial cells. These results suggest that SOCE participated in the cell migration of the rat dental epithelial cells through the regulation of small local $[Ca^{2+}]i$.