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High-fluoride promoted phagocytosis-induced apoptosis in a matured ameloblast-like cell line

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ABSTRACT

Endocytosis and phagocytosis are important physiologic activities occurring during ameloblast differentiation. We have previously found that excess fluoride inhibited ameloblasts endocytotic functions. Here, we hypothesized that increasing amounts of fluoride may affect ameloblast phagocytotic function during their differentiation. Using cell culture, we first induced maturation of the mouse ameloblast-like LS8 cells by treatment with exogenous retinoic acid (RA) and dexamethasone (DEX). We measured their phagocytotic activity by fluorescent microscopy using a live cell visualization station. We found that ameloblast-like LS8 cells matured with RA/DEX treatment and the increasing amounts of fluoride demonstrated the up-regulated expression of the phagocytotic marker proteins, LAMP1 and CD68. A connection between phagocytosis and apoptosis was confirmed by the increased number of phagocytotic vacuole-like structures and the heterochromatin margination phenomenon observed in the RA/DEX with NaF treatment group. The increase in albumin uptake by ameloblasts was confirmed using whole organ culture of incisor tooth germs. Here, in fluoride treated tooth germs, mature canonical ameloblasts showed greater amounts of albumin uptake, which was accompanied by decreased expression of the anti-apoptosis marker, Bcl-2 along with up-regulated expression of CD68. From these observations, we inferred that high doses of fluoride may cause apoptosis by increasing the phagocytosis of protein particles in mature-stage ameloblasts and loss of Bcl-2 signals might be involved in this process.

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Abbreviations: RA, retinoic acid; DEX, dexamethasone; LAMP1, Lysosomal-associated membrane protein 1; CD68, Cluster of differentiation 68; Bcl-2, B-cell lymphoma-2.

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1. Introduction

Ameloblasts carry out both secretory and degradative functions on enamel matrix proteins throughout enamel formation. Ameloblasts in the temporally latter stage of enamel maturation stage uptake aged enamel proteins.[1–4] Sasaki observed that maturation stage ameloblasts absorbed soluble proteins from their ruffled border by pinocytotic vacuoles or pinocytotic coated vesicles.[5] In our previous study, we found that millimolar concentrations of fluoride reduced chloride-channel-dependent receptor-mediated endocytosis in porcine ameloblast-like PABSo-E cells, and stimulated an acidic intracellular environment.[6] Multiple vesicular endocytic trafficking pathways can coexist simultaneously in the same cell type, including ameloblasts.[7,8] Acidification is an important marker of phagosome maturation and the phagocytosed material can be degraded only when the phagosome has achieved a sufficiently low pH.[9] It has also been shown that intracellular acidification can enhance phagocytosis in neutrophils.[10] High levels of fluoride have been shown to directly affect ameloblast physiology.[11] Using ameloblasts from enamel organs in their maturation stage, fluoride treatment induces ER stress and increases SIRT1 expression, a key regulator of autophagy during cell stress.[12,13] However, the effect of high amounts of fluoride on the function of ameloblast phagocytosis remains unknown. Moreover, there has not been a definite conclusion reached concerning the effect of fluoride on the function of phagocytosis in other cell types, and some reported results have been controversial. For example, it was reported that fluoride stimulated phagocytosis in ploymorphonuclear leucocytes via increasing phagocytotic susceptibility to bacteria or via direct stimulation of phagocytes.[14] However, another group reported that fluoride inhibited human neutrophil phagocytosis.[15]

In our study, we chose two approaches to the issue of phagocytotic function in ameloblasts. The first is based on mouse ameloblast-like LS8 cells as experimental targets where we induced their maturation with retinoic acid (RA) and dexamethasone (DEX) and also exposed them to NaF. Second, using whole tooth germs held in organ culture and treated with high fluoride we examined the phagocytosis characteristics among canonical ameloblasts from the maturation stage of differentiation. Using albumin uptake by cells in both experimental systems, we found that high doses of fluoride enhanced phagocytotic function which led to apoptosis.

2. Materials and methods

2.1. Cell culture

LS8 cells, an SV40-immortalized mouse ameloblast-like cell line, were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C in a 5% CO_2 humidified atmosphere.[16] In order to induce maturation, cells were treated with 20 μg/ml retinoic acid (RA) and 10^{-7} M dexamethasone (DEX) for 48 h.[6,17] In the following experiments, 2 mM NaF treatment for 48 h is identified as the "NaF^+" group, treatment with RA and DEX for 48 h is identified as the "RA/DEX^+" group and when all treatments are deployed, the cells are identified as RA/DEX^+NaF^+.

2.2. Phagocytosis assays with live cell station microscope

Cells were cultured with 0.1 mg/ml FITC-albumin in PBS with 1.36×10^{-3} M Ca^{2+} and 1.02×10^{-3} M Mg^{2+} at 37 °C, in a 5% CO_2 live-cell station for up to 45 min. Real-time images were captured every 30 s using a FluoView FV1000 Confocal Microscope (Olympus Corporation, Japan). Internalization of FITC-albumin particles (>500 nm, green fluorescent signature) was scored as a phagocytotic event. The numbers of cells involved in phagocytosis and the time point when cells began to phagocytize FITC-albumin were recorded and compared among treatment groups. At least 50 cells were randomly sampled and scored for each group and repeated in triplicate.

2.3. Immuno-detection of phagocytosis markers

Cells were seeded in 24-well plates with 10 mm diameter coverslips. After being induced with RA/DEX and/or NaF, the cells were fixed with 4% formaldehyde for 15 min and permeabilized with 0.03% Triton X-100 in PBS for 30 min. Nonspecific binding was blocked with 10% non-immune serum for 30 min at room temperature. Cells were incubated with goat anti-mouse LAMP1/CD107a antibody (R&D Systems, USA)(1:1000 dilution) and rabbit anti-mouse CD68 antibody (EPITOMICS, USA) (1:2500 dilution) in PBS containing 3% bovine serum albumin at 4 °C overnight and washed extensively with PBS. The primary antibody was detected by incubation with FITC conjugated rabbit anti-goat IgG (CWBIO, Beijing, China) (1:100 dilution) or Cy3 conjugated goat anti-rabbit IgG (CWBIO, Beijing, China) (1:100 dilution) at 37 °C for 1 h, and washed extensively with PBS. Nuclei were counterstained with Hoechst 33342 (Sigma) (1:1000 dilution) for 10 min. Images were captured using a FluoView FV1000 laser confocal microscope as described. A Leica DM6000B fully automated upright microscope system (Leica Microsystems, Germany) was used to analyze the immunodetection signals indicative of expressed protein levels. At least 50 randomly selected cells were ascertained for each group and performed in triplicate. The intensity of immunodetected signal was measured and analyzed with Image J version 1.42 software (NIH, USA).

2.4. TEM analysis

Cells were treated with RA/DEX and/or NaF for 48 h and collected by treatment with 0.25% trypsin digestion and recovered by low-speed centrifugation. Cells were fixed with 2.5% glutaraldehyde at 4 °C for 3 h and 1% osmium tetroxide at 4 °C about 1 h, respectively. The cells were dehydrated by emersion in an increasing gradient of ethanol, and embedded with Epon 812. Ultrathin sections of 50 nm were prepared, stained with uranyl acetate and lead citrate, and observed by transmission electron microscopy using a JEOL TEM operated at 200 kV (JEM 2000, JEOL, Japan).