Published in final edited form as:

Arch Oral Biol. 2015 January ; 60(1): 84–90. doi:10.1016/j.archoralbio.2014.09.001.

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 affects 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 with increasing amounts of fluoride exposure phagocytosis by LS8 cells matured by RA/DEX treatment and with fluoride (NaF) and only fluoride treated with LS8 cells demonstrating upregulated amounts of the phagocytotic protein markers, LAMP1 and CD68. The 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 NaF treated group. The increase in albumin uptake by ameloblasts was confirmed using whole organ culture of incisor tooth germs. Here, in fluoride treated mature ameloblasts, we observed higher albumin uptake, which was accompanied by decreased amounts of the apoptosis marker, Bcl-2 and up-regulated expression of CD68. From these observations, we infer that high doses of fluoride may cause apoptosis by increasing the uptake of large protein particles in matured ameloblasts and loss of Bcl-2 signals might be involved in this process.

Keywords

enamel; fluoride; endocytosis; phagocytosis; maturation

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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 proteins¹⁻⁴. Sasaki observed that maturation ameloblasts absorbed soluble proteins from the ruffled border by pinocytotic vacuoles or by pinocytotic coated vesicles⁵. 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 environment⁶. Multiple ways of vesicular endocytic trafficking can coexist simultaneously in the same cell type including ameloblasts^{7,8}. Acidification is an important marker of phagosome maturation and the phagocytosed particles can be degraded only when the phagosome achieves a sufficiently low pH⁹. It has also been shown that intracellular acidification can enhance phagocytosis in neutrophils¹⁰. High levels of fluoride have been shown to directly affect ameloblast physiology¹¹. In 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. Also, there has not been a definite conclusion about the effect of fluoride on the function of phagocytosis in other cell types, and some results were controversial. It was reported that fluoride stimulated phagocytosis in polymorphonuclear leucocytes via increasing the phagocytes susceptibility to bacteria or via direct stimulation of phagocytes¹⁴. However, another group reported that fluoride inhibited human neutrophil phagocytosis¹⁵. In this study, we chose two approaches to the issue of phagocytotic function in ameloblasts. The first is based on mouse ameloblast-like LS8 cells as targets where we induced the maturation of LS8 cells with retinoic acid (RA) and dexamethasone (DEX). Second, we examined the phagocytosis characteristics among ameloblasts from the maturation stage of differentiation using whole tooth germs held in organ culture and treated with high fluoride. Using albumin uptake in cells from the LS8 ameloblast-like cell line and ameloblasts from incisor germs, we found that high doses of fluoride enhanced phagocytotic function which lead to cell 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), 100U/ml penicillin and 100mg/ml streptomycin at 37°C in a 5% CO₂ humidified atmosphere¹⁶. In order to induce LS8 cells maturation, cells were treated with 20µg/ml retinoic acid (RA) and 10⁻⁷M dexamethasone (DEX) for 48 hours^{6, 17}. In the following experiments, 2mM NaF for 48 hours is identified as the "NaF⁺⁺" group and the combined treatment of RA and DEX is identified as the "RA/DEX⁺⁺" group.

2.2 Phagocytosis Assays with Live Cell Station

Cells were cultured with 0.1mg/ml FITC-albumin in PBS with $1.36 \times 10^{-3} \text{M}$ Ca^{2+} and $1.02 \times 10^{-3} \text{M}$ Mg^{2+} at 37°C , in a 5% CO_2 live-cell station for up to 45 minutes. Real-time images were captured using a FluoView FV1000 Confocal Microscope (Olympus Corporation, Japan) every 30 seconds. Internalization of FITC-albumin green particles ($>500\text{nm}$) was scored as phagocytosis. 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. 50 cells were randomly sampled and scored for each group.

2.3 Immunofluorescence Assays

Cells were seeded in 24-well plates with 10mm diameter coverslips. After being induced with RA/DEX and/or NaF, induced cells were fixed with 4% formaldehyde for 15 minutes and permeabilized with 0.03% Triton X-100 in PBS for 30 minutes. Nonspecific binding was blocked with 10% serum for 30 minutes 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, followed 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 hour, and washed extensively with PBS. Nuclei were counterstained with Hoechst 33342 (Sigma) (1:1000 dilution) for 10 minutes. Images were captured using a FluoView FV1000 laser confocal microscope as elsewhere. A Leica DM6000B fully automated upright microscope system (Leica Microsystems, Germany) was used to analyze the immunodetection signals indicative of expressed protein levels. 50 randomly selected cells were ascertained for each group. The intensities of positive immunodetection staining were 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 48hours 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 hours and 1% osmium tetroxide at 4°C about 1 hours, respectively. Then dehydrated by gradient ethanols, later embedded with Epon812. 50nm ultrathin sections were prepared, stained with uranyl acetate and lead citrate, and observed with transmission electron microscopy using a JEOL TEM operated at 200 kV (JEM 2000, JEOL, Japan).

2.5 Tooth Germ Organ Culture Assays

Incisor germs were dissected from newborn mice, maintained in organ culture at the gas/media interface on a water jacketed CO_2 injected humidified incubator and treated with either 2mM NaF or 2mM NaCl (control) for 48 hours. The tooth germs were incubated with 0.2 mg/ml FITC-albumin in PBS with $1.36 \times 10^{-3} \text{M}$ Ca^{2+} and $1.02 \times 10^{-3} \text{M}$ Mg^{2+} at 37°C in 5% CO_2 humidified atmosphere for 30 minutes, and rapidly washed twice with acid stripping solution to block further uptake (50mM glycine, 2M urea, 30g/L BSA, 100mM NaCl, pH 2.5) and fixed with 4% freshly prepared paraformaldehyde. Nonspecific binding

was blocked with 10% goat serum for 30 minutes at room temperature. The tooth germs were incubated with rabbit anti-human/mouse Bcl-2 antibody at 4°C overnight. After being washed with PBS for three times, the samples were treated with Cy3 conjugated goat anti-rabbit IgG for 1 hour at 37°C. Images were captured using a FluoView FV1000 Confocal Microscope.

2.6 Dental Fluorosis Model

Female C57Black/6 mice at the time of weaning (circa 3 weeks old) were obtained from the Animal Center of the Fourth Military Medical University (FMMU). Animals were housed under controlled conditions (21°C and 12h light: 2h dark). Mice were fed ad libitum on one commercial diet: a control group in which animals received only distilled water for drinking or treatment group, where animals received distilled water with 50 ppm NaF in the drinking water for 2 months. All experimental procedures were approved by the Institutional Review Boards of the School of Stomatology, FMMU. At the end of 2 months, the whole mandible was dissected, skinned and fixed with 4% PFA overnight with decalcification in osmotically balanced 10% EDTA, pH 7.2. General histological procedures were used to produce paraffin sections at 5µm thickness. Standard hematoxylin and eosin staining of sections was performed. Immunofluorescence detection of CD68 and Bcl-2 protein expression was carried out as described above.

2.7 Statistic

Data were expressed as the mean $\pm$ SEM. Differences between experimental groups and the control groups were tested for significance by *Dunnett-t* test. *P* values less than 0.05 were considered significant.

3. Results

3.1 Fluoride Facilitates Phagocytosis in LS8 Cells

In a previous study, we plotted ameloblast-like LS8 cell death to increasing fluoride doses¹⁷, and from that published data, we established that 2mM fluoride was optimal. To induce a state of maturation in the ameloblast-like LS8 cells, we used a treatment cocktail consisting of retinoic acid and dexamethasone^{6, 17}. We used a live cell station to dynamically observe phagocytosis among the four different cell treatment groups: two control groups of ameloblast-like LS8 cells without or with NaF (RA/DEX⁻ NaF⁻, RA/DEX⁻ NaF⁺) and two groups of RA/DEX induced maturation of LS8 cells, without or with NaF treatment (RA/DEX⁺ NaF⁻ and RA/DEX⁺ NaF⁺) (Fig.1). When first introduced into the microscope live cell stage, FITC-albumins appeared as punctate green fluorescent particles that bind to LS8 cell membrane or remain suspended in the medium. With the passage of several minutes, the FITC-albumin particles were gradually phagocytized (Fig 1). Matured LS8 cells, those treated with RA/DEX⁺ revealed the greatest gains in phagocytic activity compared to the non-matured group lacking RA/DEX treatment. Matured cells that were also treated with fluoride (RA/DEX⁺ NaF⁺) revealed the greatest gain in phagocytotic activity for the FITC-albumin, reaching a peak of phagocytosis where more than 40 cells (~80%) were scored as positive for FITC-albumin. Noteworthy is the observation that the RA/DEX⁺ matured cells reached a steady-state plateau for phagocytotic functions within 15

minutes. The video shows the cell dynamics of phagocytosis for each of the four treatment groups is provided in Supplemental data (S Video 1, 2, 3 and 4).

3.2 Fluoride Up-regulates Phagocytosis Markers in LS8 Cells

LAMP1 and CD68 are two markers of phagocytosis^{14,15}. Immunofluorescence staining revealed that LS8 cells expressed both markers (Fig 2). After 48 hours RA/DEX induced-maturation with or without 2mM NaF treatment of LS8 cells, the LAMP1 protein level in the experimental groups were significantly increased compared to detection levels observed in the control group ($P<0.05$). LAMP1 expression was greatest in the RA/DEX⁺ treated LS8 cells (Fig 2B). The expression of CD68 was increased in the RA/DEX⁻NaF⁺ and the RA/DEX⁺NaF⁺ versus the control group ($P<0.05$), with the greatest CD68 expression levels observed in the RA/DEX⁻NaF⁺ treated LS8 cells (Fig. 2D).

3.3 Ultrastructural Features of High Fluoride Treated LS8 Cells

There were abundant amounts of rough endoplasmic reticulum in the cytoplasm of the control cells. The RA/DEX treated cells showed less endoplasmic reticulum and some phagocytotic vacuole like structures. Irregular enlarged cyst structures were observed in the NaF treated cells. The RA/DEX⁺NaF⁺ group cells displayed the plenty of phagocytotic vacuole like structures in the cytoplasm and the heterochromatin margination in the nucleus (Fig. 3A).

3.4 Phagocytosis were Accompanied by Apoptosis in High Fluoride Treated Ameloblasts

In the incisor germ albumin uptake experiment, stronger green FITC-albumin fluorescence were observed within the anterior ameloblasts in mature stage (Fig. 3B1) than within the posterior ameloblasts in secretory stage (Fig. 3B2 and Fig. 3C). Correspondingly, the expression of anti-apoptotic Bcl-2 protein (red fluorescence) was also higher in the maturation stage cells than in the secretory stage cells (Fig. 3D). 2mM NaF treatment increased the uptake of albumins but decreased the expression of Bcl-2 in the maturation stage cells (Fig. 3B3, 3C and 3D). 2mM NaF treatment did not significantly change the uptake of albumins but still decreased the expression of Bcl-2 in the secretory stage cells (Fig. 3B4, 3C and 3D).

We further confirmed the relationship of phagocytosis and apoptosis *in vivo*. We created an animal model of dental fluorosis and detected the markers of phagocytosis and apoptosis in the incisors. The immunofluorescence staining analysis showed that Bcl-2 was down-regulated and CD68 was little up-regulated in the fluorosis incisors (Fig. 3E).

4. Discussion

Our group had previously demonstrated that LS8 cells belonged to secretory ameloblasts and RA/DEX treatment induced LS8 cells to differentiate into the transitional/early maturation stage.¹⁷ In order to observe the effect of high fluoride on phagocytosis function in different stages of ameloblasts, we used live cell station to observe the phagocytosis of FITC-albumin in ameloblasts dynamically and focused on the green particles larger than 500nm size which was a sign of phagocytosed particles.^{8,18} We found that the RA/DEX

treated cells performed more active phagocytosis function than the control cells. Fluoride treatment could enhance phagocytosis function in both secretory stage ameloblasts and transitional/early maturation stage ameloblasts. The cells in RA/DEX+NaF⁺ group showed the most active phagocytosis function among the four groups and more than 40 cells began to phagocytose FITC-albumin within 15 minutes. These results indicated that fluoride greatly facilitated the phagocytosis function in mature ameloblasts.

We further detected two important phagocytosis markers LAMP1 and CD 68 in the four groups. The increased protein levels of LAMP1 and CD68 in RA/DEX and/or NaF treatment cells also indicated that both RA/DEX and NaF may strengthen phagocytosis function in LS8 cells. Of note, LAMP1 and CD68 protein expressions of the RA/DEX+NaF⁺ group were not the highest among the four groups, which seemed in contradiction with live cell analysis. LAMP1 and CD68 are abundant in late endosomes, lysosomes and mature phagosomes.^{19, 20} In the RA/DEX+NaF⁺ group, phagocytosis function was active and large numbers of phagosomes were formed constantly. These newly formed phagosomes need a short period to acquire LAMP1 and CD68. Immature phagosomes may explain the decreased LAMP1 and CD68 protein levels in the RA/DEX+NaF⁺ group. Furthermore, the decreased LAMP1 intensity in mature LS8 cells may also result from cell damage due to high fluoride levels.

Phagocytic vacuole like structures within TEM images also indicated that RA/DEX and/or NaF treatment cells possessed stronger phagocytosis function and that RA/DEX treatment cells might be more sensitive to high doses of fluoride exposure. The different responses to overdoses of fluoride between the control and the RA/DEX induced cells suggested that fluoride might easily target ameloblasts in the transitional or the early maturation stage. This was consistent with the *in vivo* data that the late secretory / transitional stage ameloblasts were more sensitive to short exposure to high dose of fluoride.²¹ Interestingly, we observed that phagocytic vacuole like structures and the heterochromatin margination phenomenon (the characteristic morphological features of apoptosis) appeared simultaneously in the RA/DEX+NaF⁺ cells. Phagocytosis of pyogenic bacteria can stimulate phagocytes to undergo apoptosis. In macrophages, apoptosis was observed at 8–16 hours after bacterial ingestion.²² Modulation of phagocytes apoptosis by bacteria has emerged as a mechanism of infection pathogenesis.²³ Thus, the TEM images suggested us the possibility that phagocytosis results in apoptosis in ameloblasts.

In our previous study, we discovered that high amounts of fluoride may facilitate apoptosis in matured ameloblast-like LS8 cells by down-regulating Bcl-2 expression.¹⁷ Thus, we selected Bcl-2 as a marker of apoptosis. The mouse incisor is a good model to study the ameloblasts of different differentiation stages. The mature cells located in the anterior part and the secretory cells located in the posterior part. By the albumin uptake analysis in incisor germs, we found that the uptake of albumin was strengthened at the maturation stage part than at the secretory stage part in all tooth germs. The strengthened uptake activity was accompanied by the down-regulation of Bcl-2 expression at the maturation stage part of high fluoride treated tooth germs; however, the Bcl-2 protein was up-regulated at the maturation stage part than at the secretory stage part in control tooth germs. Strengthened phagocytosis function accompanied down-regulation of Bcl-2 expression in mature ameloblasts but not in

secretory ameloblasts. At the same time, the high expression of phagocytosis marker CD68 and the low expression of anti-apoptotic Bcl-2 in the fluorosis incisors were consistent with the albumin uptake results in incisor germs. Bcl-2 has a broad anti-apoptotic effect and can block phagocytosis-induced cell death. It has been demonstrated that Bcl-2 protected macrophages and granulocytes against phagocytosis-induced apoptosis.²⁴ The down-regulated Bcl-2 may indicate weakened protective function against phagocytosis-induced cell death.

To sum up, fluoride induces apoptosis and phagocytosis function simultaneously in matured ameloblast-like LS8 cells. Fluoride may enhance phagocytosis which finally results in Bcl-2 related apoptosis (Fig. 4).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by the Foundation of National Natural Science Foundation of China (81271116, 81070819, 31070835), and from the National Institute for Dental and Craniofacial Research DE06988. We thank the Equipment Center, PLA Institute of Stomatological Research for the technique support. We thank Dr. Rong Zhang and Mr. Geng Cheng for the help with the animal experiments.

Abbreviations

RA	tretinoic acid
DEX	dexamethasone
LAMP1	Lysosomal-associated membrane protein 1
CD68	Cluster of differentiation 68
Bcl-2	B-cell lymphoma-2

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Figure 1.

Effects of RA/DEX and 2mM NaF on phagocytotic function LS8 cells using live-cell station image analysis. The X-axis represents time points. The Y-axis represents the total numbers of cells that internalized large green particles (diameters>500nm). RA/DEX and 2mM NaF treatment increased the number of cells engaged with FITC-albumin phagocytosis. The RA/DEX⁺NaF⁺ group revealed the greatest number of cells engaged in phagocytosis process.

Figure 2.

Effects of RA/DEX and 2mM NaF on the expression of phagocytosis markers in LS8 cells. (A) Laser confocal scanning microscopic images identify LAMP1 protein (green) by immunodetection with nuclei marked by Hoechst-33342 dye uptake (blue); 1) RA/DEX⁻NaF⁻; 2) RA/DEX⁺NaF⁻; 3) RA/DEX⁻NaF⁺; 4) RA/DEX⁺NaF⁺. Scale bar. 10μm. (B) Image analysis software was used to plot and display the intensity of LAMP1 protein that was found to be significantly greater ($*P < 0.05$) in each of the three treatment groups (RA/DEX⁺NaF⁻; RA/DEX⁻NaF⁺; RA/DEX⁺NaF⁺) than when compared to controls (RA/DEX⁻NaF⁻). (C) Immunofluorescence staining results for CD68 (red) and nuclei identified by Hoechst-33342 staining (blue) imaged by laser confocal scanning microscopy. 1) RA/DEX⁻NaF⁻; 2) RA/DEX⁺NaF⁻; 3) RA/DEX⁻NaF⁺; 4) RA/DEX⁺NaF⁺. Scale bar = 10μm (D) Image analysis software was used to plot and display the intensity of immunodetected CD68 (red) which was statistically greater ($*P < 0.05$) in the RA/DEX⁻NaF⁺ and RA/DEX⁺NaF⁺ treatment groups when compared to control.

Figure 3.

Connection between phagocytosis and Bcl-2. (A) Ultrastructural features of RA/DEX and NaF induced maturation of ameloblast-like LS8 cells. (1) In cells of the RA/DEX⁻NaF⁻ group, a well-developed rough endoplasmic (arrow) was abundant; (2) In cells of the RA/DEX⁺NaF⁻ group, less endoplasmic reticulum (arrow) was observed while phagocytic vacuole-like structures (arrow head) were identified; (3) In cells from the RA/DEX⁻NaF⁺ group, irregularly-enlarged cyst and cavity-like structures (arrow) are seen; (4) In cells from the RA/DEX⁺NaF⁺ group, peripheral chromatin condensation (arrow) and abundant phagocytic vacuoles (arrowheads) were observed. Bars=200nm. (B) Phagocytosis of FITC-albumin for ameloblast from new born mouse incisor tooth organs maintained in organ culture. Controls (1, 2) were treated with NaCl and experimental group (3, 4) were treated with NaF and imaged using laser confocal scanning microscopy. Since incisors manifests a continuous gradient of ameloblast differentiation, maturation stage (1,3) or to secretory stage (2, 4) cell could be sampled from one organ. Bars=20μm. The uptook FITC-Albumin, Bcl-2 staining, Hoechst-33342 staining were shown as green, red and blue respectively. (C) and (D) Comparison of average fluorescent intensities of FITC-albumin and Bcl-2. (E) CD68 and Bcl-2 staining of normal (control) or fluorosis (experiment) incisors results from laser confocal scanning microscope. The positive staining of CD68 and Bcl-2 were shown as red respectively. Nuclei identified by Hoechst-33342 staining (blue). Bars=30μm. **P*<0.05, ***P*<0.01 vs. NaCl group posterior. #*P*<0.05, ##*P*<0.01 vs. NaCl group anterior.

Figure 4.

Schematic cartoon for phagocytosis and apoptosis in fluoride treated ameloblast-like LS8 cells. High fluoride doses facilitate the expression of CD68 and Lamp1, markers of phagocytosis. The fluoride induced increase in phagocytotic activity is correlated with reduced amounts of Bcl-2 and may result in cell death and apoptosis.