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High-fluoride acitivates the FasL signalling pathway and leads to damage of ameloblast ultrastructure

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ABSTRACT

Objective: High fluoride can induce stress-mediated apoptosis and degradation of ameloblasts. Fas ligand (FasL) has been regarded as a key regulator in intracellular responses for stress-induced apoptosis in reproductive or cancerous cell lineages. The objective of this study is to explore the role of FasL in the regulation of ameloblast ultrastructure damage.

Design: Primary ameloblasts were isolated from the molar tooth germ of 4-day-old SD rats. The ameloblasts were incubated with 3.2 mM NaF or nothing. After incubation for different time arranging from 12 h to 72 h, ELISA was used to detected the secretion levels of FasL in the medium. Then at 48 h post treatment, the ameloblast ultrastructure was detected with Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM), and expression of apoptotic proteins and peroxidative enzymes/products were examined. Finally, a specific FasL inhibitor was applied to co-treat the ameloblasts with NaF, and the ameloblast ultrastructure was detected with TEM and SEM.

Results: The secretion of FasL was notably increased by 3.2 mM NaF treatment, and the increase reached to the peak after incubation for 48 h. High fluoride incubation damaged the ameloblast untrastructure manifesting a series of intracelluar stress responsing cell organelle destruction, and a marked increase in expression of apoptotic genes and oxidative stress. The FasL inhibitor treatment partially mitigated the untrastructure damage caused by high dose NaF.

Conlusion: High-fluoride leads to damage of the ameloblast ultrastructure through paritially acitivating the FasL signalling pathway.

1. Introduction

Ameloblasts are cells presenting in the hard outermost layer of developing teeth. Ameloblasts express a series of closely related proteins involved in amelogenesis, including amelogenin, ameloblastins, enamelins and tuftelins (Habelitz, 2015; Stephanopoulos, Garefalaki, & Lyroudia, 2005). These proteins direct the mineralization of enamel to form a highly organized matrix of rods, interrod crystal, and protein (Ruch & Karcher-Djuricic, 2013; Smith, 1979; Stephanopoulos et al., 2005). Researchers have focused on many aspects of ameloblast biology, including apoptosis, differentiation, secretion, endocytosis, phagocytosis, and calcium transport (Liu et al., 2015; Simmer et al., 2014; Shusterman, Gibson, Li, Healey, & Peng, 2014; Yang et al., 2015; Zhang et al., 2016). Dental fluorosis is a common endemic disease caused by chronic or acute high fluoride content in the drinking water or diet water, which mainly damages the ameloblasts and inhibits the development of the enamel (Cardoso et al., 2015; Leite et al., 2011). A large number of reports have indicated that high fluoride induced multiple-pathway-mediated apoptosis and degradation, and had a markedly negative influence on the secretion, endocytosis and phagocytosis of ameloblasts (Aoba & Fejerskov, 2002; DenBesten & Li, 2011; Sierant & Bartlett, 2012a; Yang et al., 2015; Zhang et al., 2016). The specific pathogenesis of dental fluorosis has not been still determined. Some recent studies revealed that excessive fluoride damaged the ultrastructure of ameloblasts, manifesting the reduction the cell organelles, the endoplasmic reticulum expansion, mitochondrial deformation, cavitation in the cytoplasm, and

Abbreviations: FasL, Fas ligand; TEM, Transmission Electron Microscopy; SEM, Scanning Electron Microscopy; TNF, tumor necrosis factor; DISC, deathinducing signalling complex; NaF, sodium fluoride; DAB, dia-minobenzidine; PVDF, polyvinylidene fluoride; ECL, chemical luminescence; TGF-β1, transforming growth factor β1; UPR, unfolded protein response; AMBN, ameloblastin; CK14, cytokeratin-14; caspase, cysteinyl aspartate specific proteinase; MDA, malondialdehyde; SOD, superoxide dis-mutase; GSH-Px, glutathione peroxidase.

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finally led to apoptotic cell morphology (Sierant & Bartlett, 2012a; Yang et al., 2015; de Moraes Ramos-Perez et al., 2014).

Fas ligand (FasL) is a type-II transmembrane protein and an important member of tumor necrosis factor (TNF) family (Takahashi et al., 1994). As a well-known apoptotic gene, FasL directly participates in the formation of death-inducing signalling complex (DISC) to activate the apoptotic signalling pathway that is called the Fas/FasL pathway (Ashkenazi & Salvesen, 2014). Fas/FasL has been regarded as a reliable target for the intervention on fluorosis in several tissues, for its predominant role in the regulation of ultrastructure damage and cell apoptosis, including bone, testis and liver (Miao, Zhang, Yang, Qian, & Zhang, 2013; Sun et al., 2002; Xu et al., 2010). For instance, selenium intervention reduced Fas/FasL expression and alleviated fluorosis-induced cell apoptosis and liver injury in fluoride-exposed rats (Miao et al., 2013). However, the role of FasL in the regulation of dental fluorosis is largely unknown.

In this study, rat primary ameloblasts were isolated and cultured. The regulatory role of FasL in the ameloblast fluorosis was investigated. We found that a relative high dose (3.2 mM) sodium fluoride (NaF) induced FasL expression in a time-dependent manner, and high dose NaF damaged the ultrastructure of rat ameloblasts via upregulation of FasL expression.

2. Materials and methods

2.1. Ethics statement

This study was approved by the Ethical Committee of Xi'an Medical University. All animal procedures described herein were approved by the Experimental Animal Center of Xi'an Medical University. The rats were monitored in their home cages where they were given food and water ad libitum in a humidity and 12-h light-dark cycle controlled room. After experimentation, to minimize their pains without drugs, the mice were rapidly euthanized by cervical dislocation and decapitation by an experienced animal handler.

2.2. Isolation, culture and identification of rat primary ameloblasts

Primary ameloblasts were isolated from the molar tooth germ of 4-day-old SD rats according to the method reported in a previous study (João & Arana-Chavez, 2003). The ameloblasts were cultured in the Dulbecco's modified Eagle's medium (DMEM HIGH GLUCOSE; Gibco, Grand Island, NY, USA) supplemented with 17% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin and 100 mg/mL streptomycin, at 37° C in a 5% CO_2-humidified atmosphere. The medium was changed every 48 h. On reaching to 80% confluence, the cells were subcultured.

For identification, the cells were grown on chamber slides (at the density of 10^4/slide). When the growth state of the cells is stable, their medium was removed. The cells were washed with PBS for 5 min × 3, and then incubated at 37 °C for 45 min. After fixed with 95% ethanol for 5 min, the cells were washed with PBS for 5 min × 3 and incubated with 0.5% Triton-X100 for 20 min. Washed with PBS for 5 min × 3, the cells were treated with 3% H_2O_2 for 10 min. Washed with PBS for 5 min × 3, the cells were sealed with 5% bovine serum albumin (BSA). After removal of the sealing fluid, the slides were incubated with rabbit anti-rat CK14 and rabbit anti-rat AMBN primary antibodies respectively for 3 h at 37 °C. Washed with PBS for 5 min × 3, the cells were stained with the diaminobenzidine (DAB) method described as elsewhere. The stained slides were washed with ddH_2O for 5 min twice and embedded with neutral resin. Finally the slides were observed with XSP-4C binocular microscope (Bingyu Optical Instrument CO., LTD., Shanghai, China).

2.3. Immunohistochemical staining for FasL in the ameloblasts

The procedure of the immunohistochemical staining was generally the same to the identification of the ameloblasts except that there is a secondary antibody incubation operation before the DAB staining.

2.4. Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM)

Cells were treated with NaF or nothing 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).

3D ultrastructures of the ameloblasts treated or untreated were checked with high-resolution SEM as the approach reported in a previous study (Nanci, Zalzal, & Kan, 1993).

2.5. Western blotting

A total of 30 μg of protein from each sample was separated on a 12% SDS-PAGE. Afterwards, proteins were transferred onto activated polyvinylidene fluoride (PVDF) membranes at 200 mA for 2.5 h. After the transfer, the membranes were blocked using 5% non-fat dried milk in TBS-T. The membranes were then incubated with primary antibodies (Abcam) respectively: anti-Caspase3 (1:400 dilution) and anti-Caspase8 (1:500). For the loading control, the membranes were probed with anti-β-actin (1:800) in TBS-T for 1 h. The membranes were then incubated with HRP-conjugated anti-rabbit secondary antibody (1:2000 dilution) in TBS-T for 1 h. All the antibody were purchased from Abcam (Cambridge, UK). Finally, the membranes were incubated with ECL chemical luminescence solution (Boehringer Mannheim, Mannheim, Germany), then exposed in a ChemiDoc XRS imaging system and analyzed with software Quantity One (Bio-Rad, Hercules, CA).

2.6. Enzyme linked immunosorbent assay (ELISA) for FasL secretion

Following treatment, the medium of the ameloblasts was collected by centrifugation (centrifugated at 1000g, for 5 min, and then the supernant was collected). FasL secretion in the supernant was examined with FasL ELISA Kit (Sigma, St. Louis, MO) according to the manufaturer's instructions.

2.7. Statistical analysis

All measured values are expressed as mean ± SEM. Student's *t*-test was used to determine the significance of differences in multiple comparisons. The level of significance was set at $P < 0.05$.

3. Results

3.1. High-fluoride induced FasL expression in rat primary ameloblasts in a time-dependent manner

Primary ameloblasts were isolated from the molar tooth germ of 4-day-old SD rats. Their morphology and marker genes were detected with microscopy and immunohistochemical staining respectively. The ameloblasts cultured for 60 h connected tightly to each other, shaped oval- or polygonal- epithelioid, and mostly