Ferritin expression in the periodontal tissues of primates

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Abstract

Ferritin, an iron-binding protein, is composed of two subunits, ferritin heavy chain and ferritin light chain. It regulates many biological functions, such as proliferation, angiogenesis, and immunosuppression. The objective of this study was to determine the expression and distribution of ferritin in the periodontal tissues of primates. First, we assessed the expression of ferritin in primary cultured cells isolated from human periodontal tissues using the polymerase chain reaction and immunofluorescent staining in vitro. Second, we investigated the expression and distribution of ferritin in the periodontal tissues of Macaca fascicularis, human gingival tissues, and human gingival carcinoma tissues using immunohistochemistry in vivo. Both protein and mRNA of ferritin were constitutively present in human primary cultured cells, including those from the dental apical papilla, periodontal ligament, dental pulp, and gingival epithelium, as well as gingival fibroblasts. In M. fascicularis tissues, the immunohistochemical staining was particularly strong in blood vessel and mineralizing areas of the dental pulp and periodontal ligament. Ferritin heavy chain exhibited specific immunopositivity in the stratum basali of the epithelium in human gingival tissue, and strong immunostaining was found in peripheral regions of gingival carcinoma sites. Ferritin is constitutively present and widely distributed in the periodontal tissues of primates. Ferritin may play roles in epithelial proliferation, vascular angiogenesis, and mineralization in these tissues.

Introduction

Iron is essential for biological processes such as cell proliferation, energy production, oxygen transport, electron transport, and DNA synthesis.1 Ferritin is major cellular iron-storage protein, composed of ferritin light chain (FTL) and ferritin heavy chain (FTH). As a component of regulatory network that maintains iron homeostasis, ferritin releases iron via ferritinophagy and sequesters excess iron to avoid damage by reactive oxygen species (ROS).2 The FTH and FTL subunits are encoded by different genes and have distinct functions: the former has ferroxidase activity and converts ferrous ions to ferric ions,3 while the latter has many carboxy groups which act as iron nucleation sites.4

Ferritin also regulates biological functions independent of iron, such as cell proliferation,5 angiogenesis,6 the epithelial-to-mesenchymal transition,7 and the induction of chemokine transduction signaling pathways.8 Furthermore, new research has demonstrated that ferritin interacts with other proteins, such as PS39 and nuclear receptor coactivator 4,10 influencing their functions and altering cellular responses. The functions of ferritin have not been thoroughly clarified.

Oral cavity is a bacteria-rich environment; and since iron is a key element for the growth, survival, and cellular processes of most bacteria,11 host and pathogen may compete for it. Ferritin expression and secretion increase during infection, hypoxia, and inflammation,12 and may act against pathogens by sequestering iron and stimulating the host immune response. Previously, we demonstrated that ferritin was higher expressed in human periodontal ligament (PDL) tissues than in the other human tissues examined, including heart, skin, kidney, bone marrow, spleen, testis, lung, liver, skeletal muscle, brain, thymus.13 In human oral tissues, different mesenchymal stem cells (MSCs) are obtained to treat bone defects, spinal cord injury, and inflammatory diseases.14,15 However, the expression, distribution, and biological functions of ferritin in periodontal tissues have few reports. Human oral-derived cells are easily harvested.16 We assessed the expression of ferritin in primary cultured cells isolated from human periodontal tissues using the polymerase chain reaction (PCR) and immunofluorescent staining in vitro firstly. The anatomy and physiology of M. fascicularis are similar to humans. Gingiva is a part of periodontal tissues, and human gingival carcinoma tissues in vivo. To date, no studies have been carried out to reveal the localization and expression of ferritin in periodontal tissues of primates. This study demonstrated that ferritin was constitutively and widely expressed in the cells of periodontal tissues which played roles in proliferation, vascular angiogenesis, inflammation, and mineralization. Ferritin may be a new biomarker and therapeutic target in the pathogenesis and treatment of periodontitis and oral cancer.

Conflict of interest: The authors report no conflicts of interest related to this study.

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Materials and Methods

Ethics statement

This research protocol was approved by the Review Board and the Ethics Committee of Peking University School and Hospital of stomatology (PKUSSIRB-2011007), and was approved by the Experimental Animal Welfare Ethics section of the Peking University Biomedical Ethics Committee (Protocol LA2008-006). All clinical samples were acquired from patients who received treatment in the Peking University Hospital of Stomatology, agreed to the use of their specimens for research purposes, and gave written informed consent.

Cell culture

Human dental apical papilla cells, PDL cells, human gingival fibroblasts, human dental pulp cells, and human gingival epithelial cells were isolated and passaged according to the methods previously described. Briefly, the apical papilla, according to the methods previously described, was obtained from relatively young volunteers. The proliferation capacity of gingival epithelial cells was the poorest among the four cell types, the smallest sample, so the success rate of PDL cells (PDLCs) was the lowest at ~20%. Apical papilla, PDL, pulp, and gingival fibroblasts were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% (v/v) fetal bovine serum (FBS), 1% (v/v) antibiotics (penicillin G and streptomycin; Gibco). Gingival epithelial cells were maintained in keratinocyte serum-free medium (ScienCell, San Diego, CA, USA). All cells were cultured in a humidified atmosphere of 5% CO2 at 37°C. After 12 h of incubation, cells in 12-well culture plates (Corning Inc., Corning, NY, USA) were attached to the wall, and the morphology of cells were fully extended. These cells were used for protein detection. We cultured cells in 6-well plates for mRNA examination when the cells reached 80% confluence after 24-48 h of incubation. Cultured cells were used between the second and fourth passages.

PCR assessment of ferritin mRNA expression in primary cells

Total RNA was extracted from the above primary cultured cells using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA), then used for cDNA synthesis with a Reverse Transcription System (Toyobo, Osaka, Japan). RNA quality was assessed as the absorbance ratios at 260 nm/230 nm and then used for cDNA synthesis with a Reverse Transcription System (Toyobo, Osaka, Japan). RNA quality was assessed as the absorbance ratios at 260 nm/230 nm and 260 nm/280 nm. The synthetic cDNA was mixed with gene-specific primers and Taq Master Mix (Solarbio Science & Technology Co., Beijing, China), then the PCR was carried out. The gene-specific primers used for PCR were as follows: FTH, forward: CAGGTGGCCAGAAGCTACCA, reverse: CCACATCATCGCGGTCAAAAG; FTL, forward: ACCATGAGCTCCAGATCGTGC, reverse: ACACATCGCGGTCGAATAG; D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward: ATGGGAAGGTGAAGGTCG, reverse: GGCTCATGTGCAACACAT. All experiments were carried out in duplicate.

Immunofluorescence assessment of ferritin protein expression in primary cells

The expression of the FTH and FTL proteins was assessed by immunofluorescence. The primary cultured cells were seeded on glass coverslips. After 12 h of culture, they were fixed in 4% paraformaldehyde at room temperature for 10 min. Then, endogenous peroxidase was blocked by 3% H2O2 at room temperature for 10 min. To block nonspecific reactions, cells were treated with 10% normal rabbit serum at room temperature for 30 min. The cells were then incubated with goat anti-human primary antibodies against FTH and FTL (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 4°C overnight. After the cells were washed three times with phosphate-buffered saline (PBS), they were incubated with rabbit anti-goat secondary antibody (Zhongshan Golden Bridge Biotechnology, Beijing, China) for 1 h at room temperature. Nuclei were counter-stained with 4’,6-diamidino-2-phenylindole (DAPI). Lastly, the coverslips were mounted on glass slides and the cells were viewed under a confocal Zeiss Axiovert 600 microscope at 488 nm (green, FTL), 510 nm (red, FTH), and 405 nm (blue, DAPI). Cellular images were captured on an LSM 5 Exciter confocal imaging system (Carl Zeiss, Oberkochen, Germany). As negative controls, normal goat IgG replaced the primary antibodies.

Figure 1. Expression of FTH and FTL in human primary cells isolated from periodontal tissues. A) mRNA expression of FTH and FTL in primary cultured apical papilla cells (HAPC) (lane 1), periodontal ligament cells (HPDLC) (lane 2), gingival epithelial cells (HGEC) (lane 3), gingival fibroblasts (HGF) (lane 4), and pulp cells (HDPC) (lane 5); GAPDH served as the internal control. B) Representative images of protein expression of FTH (red), FTL (green), and cell nucleus (blue) in primary cultured cells. Scale bar: 50 μm.
Study population

Twelve healthy participants and 12 patients with carcinoma of the gingiva were recruited from Peking University Hospital of Stomatology, China. The healthy individuals, who received crown-lengthening surgery in the Department of Periodontology, were examined to determine their clinical periodontal status. Periodontal examination included probing depth (PD ≤3 mm) using a William’s periodontal probe at six sites of each tooth, and bleeding index (BI ≤20%) after probing on two sides per tooth as previously described. Gingival carcinoma samples were collected from patients who had been diagnosed in the Department of Oral and Maxillofacial Surgery. The samples were fixed in 4% paraformaldehyde and stained with hematoxylin and eosin (H&E).

Experimental animals

Three adult *M. fascicularis* (long-tailed macaque) males were used in this study (Laboratory Animal Center of Academy of Military Medical Sciences), and had been used in earlier research in our laboratory. In brief, the monkeys were 5.5-6.0 years old and weighed 5.1-5.5 kg. After euthanasia with an overdose of ketamine hydrochloride, the mandibular premolars and molars were dissected. Alveolar bone surrounding the teeth was included. These specimens were fixed in 10% paraformaldehyde, demineralized in buffered 10% EDTA at 37°C, dehydrated, and embedded in paraffin. Mesic-distal sections parallel to the dental long axis were cut at 5 μm on a microtome. Some sections were stained with H&E.

![HE](image1.png) ![FTH](image2.png) ![FTL](image3.png)

<table>
<thead>
<tr>
<th>IRS (Mean±SD)</th>
<th>Odontoblastic layer</th>
<th>Pulp proper</th>
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<tr>
<td>FTH</td>
<td>10.71±2.268</td>
<td>2.79±1.369</td>
</tr>
<tr>
<td>FTL</td>
<td>9.53±2.939</td>
<td>7.76±1.393</td>
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*p*-Value 0.216

*p*-Value <0.001

Figure 2. Immunohistochemical localization of FTH and FTL in dental pulp from *M. fascicularis*. Representative sections from specimens of dental pulp. A,B) Hematoxylin-eosin staining. C,D) Immunostaining of FTH in the odontoblastic layer and pulp proper. E,F) Positive expression of FTL in the pulp core. Data are presented as mean ± SD. IRS, immunoreactive score; FTH, ferritin heavy chain; FTL, ferritin light chain; D, dentin; CP, central pulp; O, odontoblast; DPC, dental pulp cell. Scale bars: A,C,E) 50 μm; B,D,F) 20 μm.
Immunohistochemical staining of ferritin and statistical analysis

The paraffin-embedded sections were deparaffinized with xylene and rehydrated in a series of ethanol. Endogenous peroxidase was inactivated by incubation with 3% H₂O₂ at room temperature for 10 min. Antigen was retrieved by trypsin digestion at 37°C for 10 min. Then the specimens were rinsed three times in PBS and treated with 10% normal goat serum at room temperature for 30 min followed by incubation with rabbit antihuman primary antibodies against FTH and FTL (Santa Cruz Biotechnology, Inc.) overnight at 4°C. After washing three times with PBS for 5 min each, the expression and distribution of FTH and FTL were visualized using an immunohistochemistry kit and a DAB detection kit (Zhongshan Golden Bridge Biotechnology, Beijing, China). After counterstaining with hematoxylin, the sections were mounted. Images were captured on a light microscope with a digital camera (BX51/DP72, Olympus, Tokyo, Japan).

Immunoreactive score (IRS) with modification was used to evaluate immunohistochemical staining of ferritin in the odontoblast, PDLCs adjacent to the alveolar bone and cementum.²⁷ Briefly, IRS = staining intensity (SI) x percentage of positive cells (PP). SI was defined as: 0, negative; 1, weak; 2, moderate; 3, strong. PP was determined as: 0, negative; 1, 0-10% positive cells; 2, 10%-50% positive cells; 3, 50%-75% positive cells; and 4, 75%-100% positive cells. Ten visual fields of each specimen were chosen for the IRS evaluation. Statistical analyses were performed using SPSS Statistics 20.0 software and carried out using t-test for testing two groups. The value of P<0.05 was considered statistically significant.
Results

Ferritin expression was constitutive in human primary cells isolated from periodontal tissues in vitro

The total RNA from apical papilla, PDL, pulp, and gingival epithelial cells and fibroblasts was analyzed by RT-PCR. The results revealed that FTH and FTL mRNA transcripts were strongly expressed in all these cell-types (Figure 1A). Cell immunofluorescence assays of FTH and FTL protein confirmed the PCR results (Figure 1B). The immunostaining of FTH and FTL was mainly localized in the cytoplasm. Ferritin was most abundant in apical papilla, PDL, and gingival epithelial cells followed by gingival fibroblasts and pulp cells.

Ferritin was widely distributed in periodontal tissues of M. fascicularis

Although we had shown that ferritin was present in these cells, its distribution in periodontal tissues needed to be systematically examined, because cells in different sites perform different functions. Completely healthy human periodontal tissues are difficult to obtain, while non-human primates are appropriate experimental models for research, because their anatomy and physiology are similar to humans. Microscopic analysis of periodontal tissues showed that specimens reacted to anti-FTH and anti-FTL antibodies. In detail, dental pulp, the PDL, alveolar bone, and bone marrow were positively stained for FTH and FTL.

We used immunohistochemical assays to determine the distribution of FTH and FTL in the dental pulp of M. fascicularis. The results showed that FTH staining was concentrated in the odontoblastic layer lining the outermost layer of dental pulp, and the staining of FTH in the pulp core was weaker than odontoblastic layer (Figure 2D). Meanwhile, both pulp core and odontoblastic layer were positive for FTL (Figure 2F). In the PDL, the immunoreactivity of FTH was more positive in the PDLCs adjacent to alveolar bone and cementum (Figure 3D), while FTL expression was positive in all PDLCs and VECs (vascular endothelial cells) of the PDL (Figure 3F). In the root furcation, both FTH and FTL expression were present in the osteocytes of alveolar bone (Figure 4D,F). In the bone marrow, adipocytes and fibroblasts lining the marrow center and osteoblasts lining the marrow cavity showed strong positive staining for FTH and FTL (Figure 5D,F).

Discussion

To the best of our knowledge, this is the first study to reveal the localization and expression of ferritin in the periodontal tissues of primates. Investigation of human primary cultured cells provided evidence that ferritin is constitutively expressed in human apical papilla cells, PDL cells, gingival fibrocytes, dental pulp cells, and gingival carcinoma tissues
val endothelial cells. Dental pulp, gingiva, and PDL are all vascularized connective tissues. After injury and inflammation, their healing potential is very beneficial. In part, this healing potential is associated with local cells which secrete factors that promote cells differentiation and neovascularization. Previous research has shown that ferritin has regulatory effects on angiogenesis. The expression of FTL in vascular endothelial cells in connective tissues deserves further investigation to determine whether it has a proangiogenic effect in the periodontal tissues. The local, abundantly expressed ferritin might contribute to healing. The ratio of FTH and FTL varies depending on tissue type and could be modified by pathological conditions. According to the immunohistochemical results, PDLCs and pulp cells expressed high levels of ferritin. Moreover, these cells which have mineralization activity like osteoblasts contain both FTH and FTL. This demonstrates that both FTH and FTL may be involved in the process of mineralization. Recently, it has been reported that ferritin, especially FTH, negatively modulates the

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![Figure 5. FTH and FTL expression pattern in bone marrow between roots of teeth from M. fascicularis. A,B) Hematoxylin-eosin staining. C-F) Adipocytes, fibroblasts, and osteoblasts show strong positive staining for FTH and FTL. FTH and FTL immunostaining in similar areas. AB, alveolar bone; AT, adipose tissue; FB, fibroblast; OB, osteoblast. Scale bars: A,C,E) 50 μm; B,D,F) 20 μm.](image)

![Figure 6. Expression of FTH in human gingival samples and gingival carcinoma tissue. Immunohistochemical analysis of the location of FTH expression in human healthy gingival tissues and gingival carcinoma tissues. As indicated by the arrows, we found strong immunexpression of FTH in basal cells of the epithelium in healthy gingiva. At the same time, peripheral regions of tumor islands and areas of inflammatory cell infiltration in tumorous connective tissues also expressed abundant FTH. Original magnification: 200X.](image)
Ferritin is strongly associated with cancer. As previous reports, serum ferritin is elevated in patients with multiple malignancies, including neuroblastoma, renal cell carcinoma, non-small-cell lung cancer. The level of serum ferritin is higher in tumor tissue than in normal tissue. Serum ferritin is also a potential tumor marker. However, the sources and functions of ferritin in various tumors are unclear. Moreover, the role of ferritin in the pathogenesis of cancer is still under investigation.

In this study, we found that serum ferritin was positively correlated with the number of cancer cells in vitro. Ferritin may be involved in the proliferation and differentiation of cancer cells. Ferritin may also be involved in the progression of cancer. Therefore, ferritin may be a potential target for cancer therapy.

References


