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. 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 basal 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 carboxylic 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 However, the expression, distribution, and biological functions of ferritin in periodontal tissues have few reports. Human oral-derived cells are easily harvested.15 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 could be obtained from patients of health and gingival carcinoma. We used immunohistochemical assays to detect the location of ferritin in periodontal tissues of M. fascicularis, human gingival 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.
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, PDL, and pulp cells were obtained from wisdom teeth extracted from young healthy donors who had no dental or periodontal diseases. Gingival epithelial cells and fibroblasts were isolated from healthy volunteers who received crown-lengthening surgery. The samples of dental pulp and gingival fibroblasts were collected from healthy volunteers who were 100% in the absence of microbial contamination. Dental apical papillae had to be obtained from relatively young volunteers. The proliferation capacity of gingival epithelial cells was the poorest among the cell types. PDL tissue from one tooth was the smallest sample, so the success rate of PDL cells (PDLCs) was the lowest at ~20%.

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. Endogenous peroxidase was blocked by 3% H2O2 at room temperature. Nuclei were counterstained with 4’,6-diamidine-2-phenylindole (DAPI). Lastly, the coverslips were mounted on glass slides and the cells were viewed under a confocal Zeiss Axiovert 650 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.

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 260 nm/280 nm. The synthetic cDNA was mixed with gene-specific primers and Taq Master Mix (Solabio Science & Technology Co., Beijing, China), then the PCR was carried out. The gene-specific primers used for PCR were as follows: FTH, forward: CAGGGTGCCAGAAC-TACCA, reverse: CCCATCATCGCGTCAAAAG; FTL, forward: ACCATGAGCTC CCAAGTCCGTC, reverse: ACACATCA

ATCGCGTGCAATAG; D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward: ATGGGGAAGGTAAGGTCG, reverse: GGGTCAATGGCGACAACT.

All experiments were carried out in duplicate.
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.22 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.23-26 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.

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

| IRS (Mean±SD) | Odontoblastic layer | Pulp proper |
|---------------|---------------------|-------------|
| FTH           | 10.71±2.268         | 2.79±1.369  | *p*-Value <0.001 |
| FTL           | 9.53±2.939          | 7.76±1.393  | *p*-Value 0.032 |

*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. 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.

| IRS (Mean±SD) | PDLCs adjacent to | PDLCs adjacent to |
|---------------|------------------|------------------|
|               | cementum         | alveolar bone    |
| FTH           | 5.44±1.617       | 6.11±1.811       | p-Value 0.252 |
| FTL           | 6.29±1.993       | 6.82±2.243       | p-Value 0.472 |
| p-Value       | 0.177            | 0.311            |

Figure 3. Immunohistochemical localization of FTH and FTL in periodontal ligament from M. fascicularis. A,B) Hematoxylin-eosin staining. C,D) Strong FTH expression in cementoblasts, osteoblasts, and adjacent periodontal ligament cells. E,F) FTL expression in periodontal ligament cells and vascular endothelial cells. Data are presented as mean ± SD. IRS, immunoreactive score; FTH, ferritin heavy chain; FTL, ferritin light chain; D, dentin; C, cementum; PDL, periodontal ligament; AB, alveolar bone; BV, blood vessel; CB, cementoblast; OB, osteoblast; PDLC, periodontal ligament cell; VEC, vascular endothelial cell. Scale bars: A,C,E) 50 μm; B,D,F) 20 μm.
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 innermost 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 4 D,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 5 D,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 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.

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Characteristic ferritin heavy chain expression in healthy human gingiva and gingival carcinoma tissues

We investigated ferritin expression in gingival tissues, which were not available for M. fascicularis. In gingival connective tissue, FTH was positive in the VECs (Figure 6). In the oral epithelium, FTH was positive in the stratum basal. The basal cells of oral epithelium are stem cells; they are immature and undifferentiated, with a marked capacity for division and proliferation. Therefore, we examined the expression of FTH in gingival carcinomas. Immunohistochemical staining showed the expression of ferritin in the peripheral areas of tumors and areas of inflammatory cell infiltration was stronger. Cellular FTH may affect the proliferation and invasion of cancer cells.
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

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.

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 immunostaining 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.
differentiation of osteosarcoma cells and human smooth muscle cells into osteoblasts via ferroxidase activity.\textsuperscript{30,31} We deduce that an autocrine or paracrine ferritin loop might be formed in the local environment to promote mineralization homeostasis in healthy tissues and enhance osteogenesis in disease sites. Further studies are needed to uncover the roles of ferritin in cytodifferentiation and mineralization.

Ferritin is strongly associated with cancer. As previous reports, serum ferritin is elevated in patients with multiple malignancies, including neuroblastoma,\textsuperscript{33} melanoma,\textsuperscript{34} renal cell carcinoma, non-small-cell lung cancer.\textsuperscript{35} The level of serum ferritin is higher, the disease is more proressive, and the survival is shorter. Serum ferritin in cancer patients consists mainly of FTH and is a potential biomarker.\textsuperscript{36} However, the sources and functions of ferritin in serum are unclear. Moreover, ferritin inhibits immune responses, increases angiogenesis, and promotes proliferation. Evidence has demonstrated that extracellular ferritin has immunosuppressive effects on myeloid cells and lymphocytes.\textsuperscript{12,13} In a mouse tumor model, the addition of ferritin increases intratumor blood vessel density significantly.\textsuperscript{37} Targeting FTH and FTL with shRNA results in growth reduction of cancer stem cells.\textsuperscript{38}

In this study, we found that FTH expression was positive in the stratum basal of oral epithelium and blood capillaries, was enhanced in the periphery of tumor sites where proliferation ability is high, and was upregulated in tumorous connective tissues where proliferation ability is high, and was enhanced in the periphery of tumor sites. FTH is a candidate marker of stem cells in gingival carcinoma and may be a source of elevated serum ferritin through secreting by tumor cells. Ferritin may be involved in the pathogenesis and development of gingival carcinoma by regulating cells proliferation, angiogenesis and immune response. The phenomenon and mechanism need further confirmation and investigation.

In conclusion, we identified that ferritin was constitutively present and widely distributed in the periodontal tissues of pri- mates and might play roles in epithelial proliferation, vascular angiogenesis, inflammation and mineralization in these tissues.

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