Original Article
Elevated expression of cyclophilin A in human periodontitis

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Received October 6, 2017; Accepted February 2, 2018; Epub March 15, 2018; Published March 30, 2018

Abstract: This study elucidated the correlation between cyclophilin A (CypA) and inflammatory infiltrating cells in human periodontitis. Western blotting, immunohistochemistry, and immunofluorescence were performed to detect the expression level of CypA in gingival tissues of human periodontitis. Healthy gingival tissues were chosen as a control. The distribution of CD3⁺, CD4⁺, CD22⁺, and CD68⁺ infiltrating cells in the gingival tissues were also detected by immunohistochemistry. Western blotting analysis and immunohistochemistry revealed significantly increased expression of CypA in human periodontitis. NF-κB p-p65 and p-IκBα expression was detected to investigate NF-κB activation. Immunohistochemistry and immunofluorescence identified that the positive-expressed CypA was localized in the infiltrating cells. CD3⁺, CD4⁺, CD22⁺, and CD68⁺ cells all could be observed in the CypA-positive infiltrating cells. The NF-κB pathway was activated in human periodontitis. In conclusion, CypA is involved in leukocyte attraction in the periodontal inflammatory response. These efforts not only highlight the pathogenesis of human periodontitis, but also signify CypA as a potential target for anti-inflammatory therapeutics.

Keywords: Cyclophilin A, periodontitis, inflammatory cells

Introduction

Cyclophilin A (CypA), a member of the immunophilin family, has peptidyl prolylcis-trans isomerase (PPlase) activity, which regulates immune-modulation, protein folding, trafficking assembly, and cell signaling [1, 2]. CypA is a highly conserved protein that is expressed in a wide range of tissues. Evidence supporting important functions for CypA in rheumatoid arthritis [3, 4], cancer [5], cardiovascular diseases [6], sepsis [7], periodontitis [8, 9] and aging [10], are gradually increasing. Recent research shows that CypA can be secreted by the infiltrating cells in response to inflammatory stimuli [11, 12]. CypA is a potent chemokine, which can directly induce leukocyte chemotaxis and contribute to the pathogenesis of inflammation-mediated diseases [13]. We presumed that elevated CypA induces more infiltrating cells to diseased sites, and then the infiltrating cells further secrete CypA to aggravate periodontal inflammatory destruction. The correlation of CypA and inflammatory infiltrating cells in human periodontitis not only elucidates the pathogenesis of human periodontitis, but also signifies CypA as a target for anti-inflammatory therapeutics [14]. Therefore, this work aimed to elucidate the distribution of CypA and its correlation with inflammatory infiltrating cells in human periodontitis.

Material and methods

Study participants and sample collection

Participants (20 males, aged 20-55 years, and mean 36 years) were enrolled into 2 groups (healthy and periodontitis groups) on the basis of the inflammatory status of gingival tissues. All studies were approved by the Institutional Review Board of Jinan Stomatological Hospital, and all patients provided voluntary informed consent to participate in the study. In the periodontitis group, gingival tissues were excised during extractions of teeth which were obtained from 10 patients with severe disease. In the healthy group, gingival tissues were collected...
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During orthodontic extractions from 10 healthy donors without systematic diseases. Each specimen was divided into two parts of approximately equal size. One part was immediately fixed in 4% paraformaldehyde and then 5 μm serial sections were made for immunohistochemistry and immunofluorescence analyses. The other part was stored in liquid nitrogen for Western blotting.

**Immunohistochemistry**

Immunohistochemical study was performed by using Streptavidin-Peroxidase kit (Zhongshan, Beijing, China) as previously described [15]. Polyclonal antibody CypA (dilution 1:100, Abcam, UK), CD3 (dilution 1:100, Abcam, UK), CD4 (dilution 1:100, Abcam, UK), CD22 (dilution 1:100, Abcam, UK), and CD68 (dilution 1:100, Abcam, UK) were applied. PBS was obtained as control.

**Immunofluorescence**

Sections were deparaffinized in xylene and rehydrated. After washing, the sections were incubated with polyclonal antibody CypA (dilution 1:100, Abcam, UK) at 4°C overnight. The sections were then incubated with rhodamine (TRITC)-conjugated goat anti-rabbit IgG (Sigma, USA) for 1 h at room temperature. Nuclei were stained with DAPI solution (Sigma, USA) for 5 min. The sections were photographed with immunofluorescence microscopy (OLYMPUS BX-60, Japan).

**Western blotting analysis**

Samples were washed by cold PBS three times respectively, and then homogenized in RIPA buffer for 30 min. Phenylmethylsulfonyl-fluoride (1 mM) was added into the buffer in advance. Protein concentrations were measured using a bicinchoninic acid assay (BCA) protein quantitative analysis assay kit (BOSHE, China); Proteins were separated on 10% SDS gels and then transferred onto polyvinylidene difluoride membranes (Millipore, USA). After blocking in 0.1% Tween 20 in Tris-buffered saline containing 5% nonfat dried milk for 1 h at room temperature, the membranes were incubated with antibodies against CypA (diluted 1:1000, Abcam), NF-κB p-p65 (1:1000, Cell Signaling Technology, USA) or p-IκBα (1:1000, Cell Signaling Technology, USA) overnight at 4°C. Before incubation with horseradish peroxidase (HRP)-labeled second antibody (Beyotime), the membranes were rinsed with...
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Expression and distribution of CypA was detected in both the periodontitis and healthy groups. Expression of CypA in the periodontitis group was higher than that of the healthy group. Positive expression was localized in the gingival epithelium of the healthy gingiva. In human periodontitis, positive staining was principally distributed in the infiltrating cells, besides gingival epithelium (Figure 1). Western blotting results also showed that expression of CypA in inflamed gingiva was higher than that of healthy donors (P < 0.05, Figure 2). According to histological observation, many inflammatory infiltrating cells could be observed in the inflamed gingival of human periodontitis (Figure 3). Expression of CypA in the inflammatory infiltrating cells was also observed by immunofluorescence. Positive expression of CypA mainly exists in the cytoplasm of the inflammatory infiltrating cells (Figure 4).

CD3⁺, CD4⁺, CD22⁺, and CD68⁺ in the CypA-positive infiltrating cells in human inflamed gingiva

CD3⁺, CD4⁺, CD22⁺, and CD68⁺ cells could all be observed in the CypA-positive infiltrating cells in the inflamed gingival tissues. CD4⁺ (T-helper/inducer) cells, CD68⁺ cells (macrophages), and CD3⁺ cells were predominant in the lamina propria and the bottom parts of the gingival epithelium. CD22⁺ B cells were arranged in clusters in the lamina propria.
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Figure 5. Expression and distribution of CD3+\textsuperscript{+}, CD4+\textsuperscript{+}, CD22+\textsuperscript{+}, and CD68+\textsuperscript{+} inflammatory infiltrating cells in human periodontitis. CD3+\textsuperscript{+}, CD4+\textsuperscript{+}, CD22+\textsuperscript{+}, and CD68+\textsuperscript{+} infiltrating cells all could be observed in the inflamed gingival tissues.

Figure 6. Quantitation of CD3+\textsuperscript{+}, CD4+\textsuperscript{+}, CD22+\textsuperscript{+}, and CD68+\textsuperscript{+} in the inflammatory infiltrating cells in human periodontitis. Positive cells were quantified by imaging five fields of view under 100-fold magnification and directly counting the number of CD3+\textsuperscript{+}, CD4+\textsuperscript{+}, CD22+\textsuperscript{+}, and CD68+\textsuperscript{+} positive cells. CD3+\textsuperscript{+} cells (T cells) comprised the major population of lymphocytes (Figure 6).

**NF-κB activation**

Western blotting results show NF-κB 65 and IκBα phosphorylation was upregulated in the inflamed gingiva to higher levels than in the healthy gingiva. Therefore, the NF-κB pathway was activated in human periodontitis (Figure 7).

**Discussion**

CypA is believed to have critical roles in regulating inflammatory responses, immunomodulation, trafficking assembly and MMPs production, and it can be secreted in response to inflammatory stimuli such as hypoxia, infection, and oxidative stress [16-18]. CypA is associated with inflammatory infiltration and alveolar bone destruction in rat experimental periodontitis. CypA can induce migration of monocyte/macrophages, lymphocytes, and neutrophils into tissues [19], and contributes to inflammatory responses through its chemotactic activity [20-22].

Our results indicated that CypA plays potential roles in the progression of human periodontitis. Positive expression of CypA mainly exists in the inflammatory infiltrating cells. The expression of CypA in the inflamed gingiva of human periodontitis was higher than that of the healthy gingiva.
Periodontitis affects a great number of patients all over the world, which leads to gingival inflammation, alveolar bone loss, and even loose teeth [23]. Different subsets of leukocytes such as monocyte/macrophages, lymphocytes, and neutrophils, are involved in the histopathogenesis of human periodontitis [24, 25]. CypA levels increase in periodontitis, but cell types expressing CypA and the function of CypA in the pathogenesis of periodontitis are not known yet. This study further elucidates the correlation of CypA and the inflammatory infiltrating cells in human periodontitis.

Inflammatory infiltrating cells are a key step in the inflammatory response. A large amount of inflammatory infiltrating cells could be observed in the inflamed gingival of human periodontitis [26]. The inflammatory infiltrating cells migrate from blood vessels into sites of inflammation, and are recognized as macrophages, lymphocytes, and neutrophils according to their shape, size, and location [27-30]. In this study, we found significant positive expression of CD3+ (T cells), CD4+ (T helper cells), CD22+ (B cells), and CD68+ (macrophages) in the infiltration of the human periodontitis. CD3+, CD4+, CD22+, and CD68+ cells all could be observed in the CypA-positive infiltrating cells in human periodontitis. Elevated CypA induced more infiltrating cells to the diseased sites, and then the infiltrating cells further secrete CypA to aggravate periodontal inflammatory destruction, and promote NF-κB pathway activation. The results showed that CypA could be targeted for anti-inflammatory therapeutics.

Although different subsets of leukocytes are recruited during the inflammatory response [31-33], synergism of CypA and different subsets of the infiltrating cells in human periodontitis still remains to be determined.

Acknowledgements

This work was supported by Natural Science Foundation of Shandong Province (ZR2017-QH007) and Science Development Program Project of Jinan (201121043) awarded to Xijiao Yu.

Disclosure of conflict of interest

None.

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