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Original Article

Importin 8 is involved in human periodontitis by the NF-κB pathway

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Abstract: This study was undertaken to investigate the roles of importin 8 (IP08) and infiltrating macrophages in human periodontitis. Gingival tissues were collected from 18 male patients with mild and severe periodontitis. IP08 expression was observed by immunohistochemistry and western blotting. CD68 expression, a marker for macrophages, was observed by immunohistochemistry and immunofluorescence. IP08 expression in mouse macrophage RAW264.7 cells stimulated by lipopolysaccharide (LPS) purified from *Porphyromonas gingivalis* was examined in vitro using small interfering RNA (siRNA). Phosphorylation of nuclear factor kappa B (NF)-κB pp65 was detected as an indicator of NF-κB activation by immunofluorescence and western blotting. IP08 expression was increased in gingival tissues with periodontitis compared with healthy ones, and the elevation rose in proportion to the severity of periodontitis. Similarly, more CD68+ infiltrating macrophages were observed in severe periodontitis tissues. LPS stimulation promoted IP08 expression in mouse macrophage cells. Knockdown of IP08 by siRNA alleviated the LPS-induced increases in tumor necrosis factor-α and interleukin-6 gene expression, as well as NF-κB activation, by decreasing the expression of NF-κB pp 65 in the nuclei of cells in vitro. In conclusion, IOP8 plays a role in human periodontitis. Targeted inhibition of IOP8 expression could effectively alleviate periodontal inflammation.

Keywords: Importin 8, CD68, periodontitis, nf-κb

Introduction

Periodontitis is common but largely preventable. Periodontitis damages gingival tissues and the alveolar bone that supports the teeth, eventually resulting in tooth loss. Infiltrating macrophages in periodontitis produce excessive amounts of inflammatory cytokines and play a role in periodontal tissue destruction [1].

Importin 8 (IP08) is an important member of protein import receptor importin β [2], which is involved in mediating the cytoplasm-to-nucleus transport of mature miRNAs [3]. A previous study has shown that IP08 participates in human pulpitis and that IP08 expression is increased in inflamed dental pulps [4]. Reports on the role of IP08 in human periodontal inflammation are rare. It is essential to elucidate the interplay between inflammatory response stimulation and IP08 expression in periodontitis. This is the first study to interpret the relationship between IP08 and macrophages in human periodontitis. Activation of lipopolysaccharide (LPS)-induced nuclear factor (NF)-κB in vitro was used to investigate the role of IP08 in the mouse macrophage cell line RAW264.7.

Materials and methods

Study participants and sample collection

Eighteen male periodontitis patients without systemic disease aged 24-60 years (mean, 36 years) were enrolled and assigned to either the severe periodontitis group (n=9) or the mild periodontitis group (n=9). Gingival tissues were excised from all participants during tooth extractions. One piece of each tissue sample was immediately fixed in 4% paraformaldehyde, from which 5-μm serial sections were made for hematoxylin and eosin staining, immunohistochemistry and immunofluorescence. The remaining samples were stored in liquid nitrogen for western blotting. The mouse macrophage RAW264.7 cell line was obtained from the

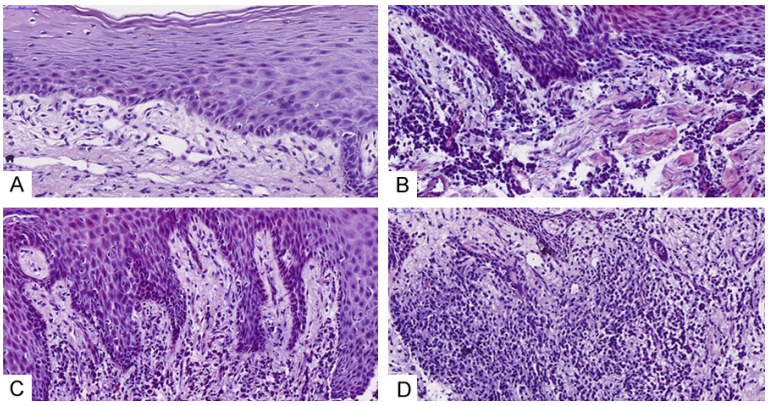

Figure 1. Histologic observation of different inflammatory infiltrates in healthy human gingiva (A), mild (B and C) and severe (D) human periodontitis.

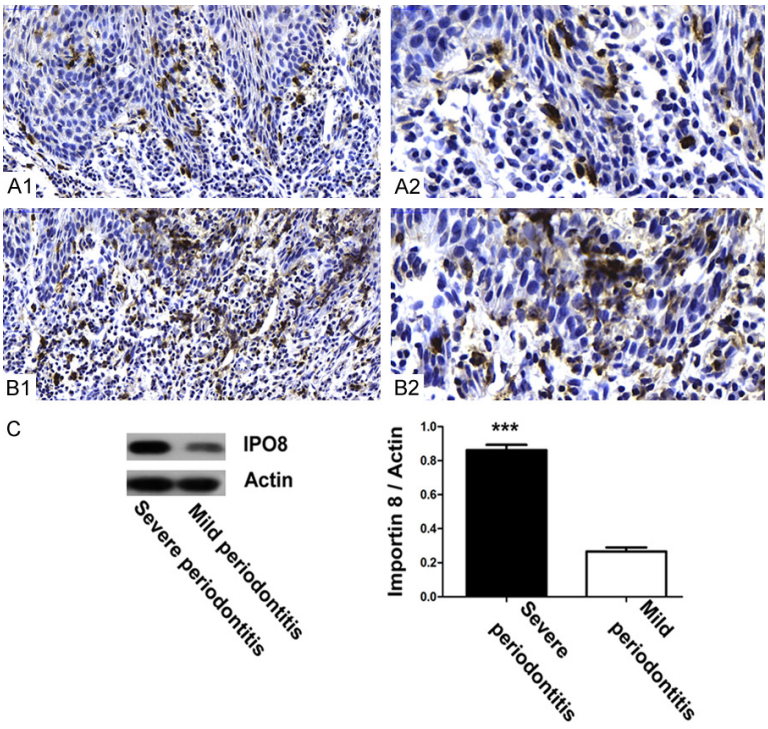

Figure 2. Expression of importin 8 in human mild (A1 and A2) and severe periodontitis (B1 and B2). Higher expression of importin 8 protein was detected in severe periodontitis, compared with mild periodontitis by western blotting (*P<0.01) (C).

American Type Culture Collection (Rockville, MD, USA). This study was approved by the Ethics Committee of Jinan Stomatological Hospital.

Immunohistochemistry and immunofluorescence

Immunohistochemistry and immunofluorescence were performed as previously described [1] using monoclonal antibody importin 8 (dilution 1:80), and polyclonal antibody CD68 (dilution 1:100), both from Abcam (UK). Mouse monocytic cells were transferred onto slides for immunofluorescence using polyclonal antibodies p65 (1:3000) and pp65 (dilution 1:3000), both from Abcam.

Knockdown of IPO8 by small interfering RNA

Macrophages were inoculated into 6-well plates and transfected with Lipofectamine® 2000 Reagent (Invitrogen) in accordance with the manufacturer's instructions. Predesigned siRNA was purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Cultures containing 2×10^5 cells each were transfected with 20 pmol of RNA. Twenty-four hours after transfection, cells were treated with LPS prepared from *Porphyromonas gingivalis* (0.5 μg/mL) for 12 hours, and subjected to real-time polymerase chain reaction (PCR) and western blotting analysis.

Real-time PCR

RNA was isolated from macrophages in accordance with the manufacturer's instructions using Trizol reagent (Invitrogen) as previously described [5] and converted to cDNA. Quantitative real-time PCR amplifications were performed in a Light Cycler (ANALYTIKJENA-qTOWER2.2, Germany) using the following primer sequences: mouse tumor necrosis factor (TNF)-α, sense 5' GCCTCCCTCTCATCAGTTCTA-3', antisense 5'-GGCAGCCTTGTCCCTTG-3'; and mouse interleukin (IL)-6, sense 5'-AGTTGTG-CAATGGCAATTCTGA-3', antisense 5'-AGGACTC-TGGCTTTGTCTTTCT-3'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous control.

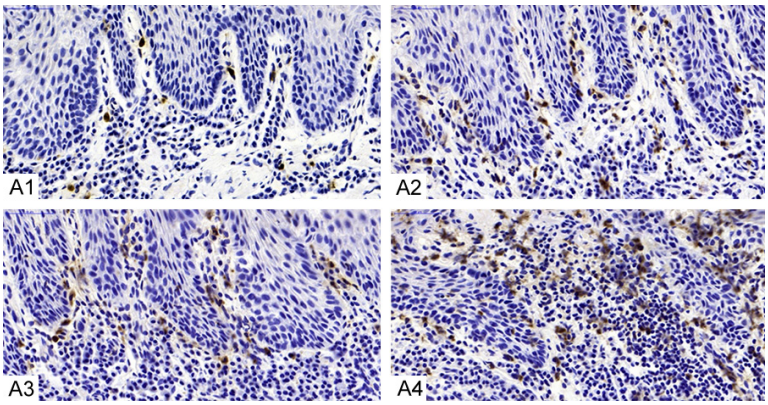

Figure 3. Expression of CD68 (macrophage maker) in human periodontitis by immunohistochemistry.

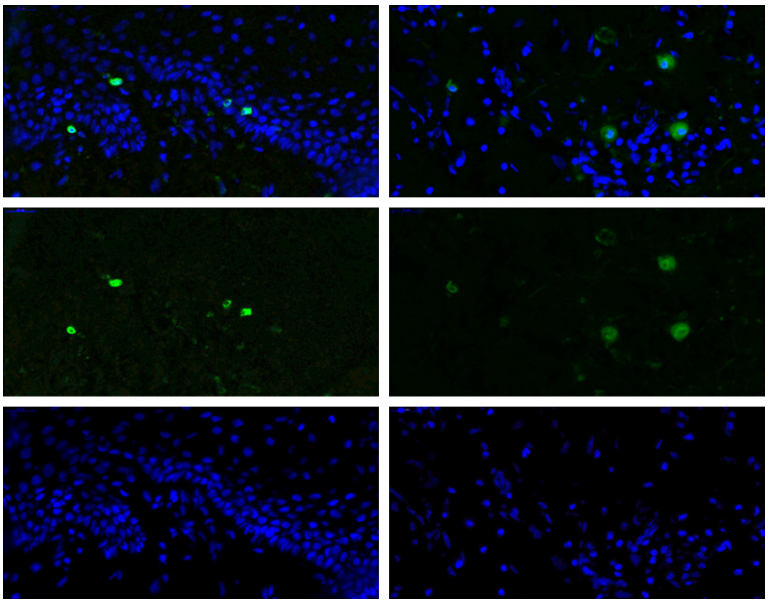

Figure 4. Expression of CD68 (macrophage maker) in human periodontitis by immunofluorescence. Positive expression mainly exists in the cytoplasm (green).

Western blotting analysis

Proteins were separated and then transferred onto membranes (Millipore, USA) as previously described [6]. The membranes were incubated with IPO8 antibody (1:500) and NF-κB pp65 (1:1000). After rinsing the membranes three times with TBST for 10 minutes each, they were incubated with horseradish peroxidase-labeled second antibody (Beyotime), and the bands were visualized on Canon films using ECL substrate solution (Millipore). β-actin (1:10000) was chosen as the loading control.

Statistical analysis

Differences were considered statistically significant at $P< 0.05$. The control group, LPS group and siIPO8 group were analyzed using one-way ANOVA analysis of variance and Student-Newman-Keuls test. Unpaired two sample t tests ($P<0.05$) were conducted for groups of two.

Results

Histologic observation of inflammatory infiltration in human periodontitis

Few infiltrating cells were observed in healthy gingiva (**Figure 1A**). Increased numbers of infiltrating cells could be found in tissue samples from patients with mild periodontitis (**Figure 1B**, **1C**), while many infiltrating cells were observed in those from patients with severe periodontitis (**Figure 1D**).

Expressions of IPO 8 in human mild and severe periodontitis

Positive staining for IPO8 was principally distributed in the infiltrating cells in human gingival tissues. We observed more inflammatory infiltrating cells and IPO8-positive cells in the severe periodontitis group (**Figure 2A1** and **2A2**) than the mild periodontitis group (**Figure 2B1** and **2B2**). We also detected a higher expression of IPO8 in severe periodontitis tissues compared with mild periodontitis tissues (*$P<0.01$) (**Figure 2C**).

Expression of CD68 in human periodontitis

Along with the increase in inflammatory infiltration, greater numbers of cells positive for CD68, a macrophage marker, were observed in periodontitis tissues. CD68-positive inflammatory infiltrating cells contributed to prolonged peri-

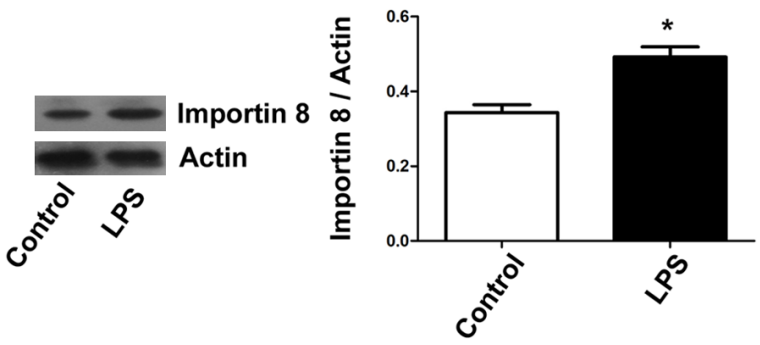

Figure 5. Expression of importin 8 in mouse macrophage RAW264.7 cells, with or without stimulation with 0.5 μg/mL P. gingivalis liposaccharide, by western blotting.

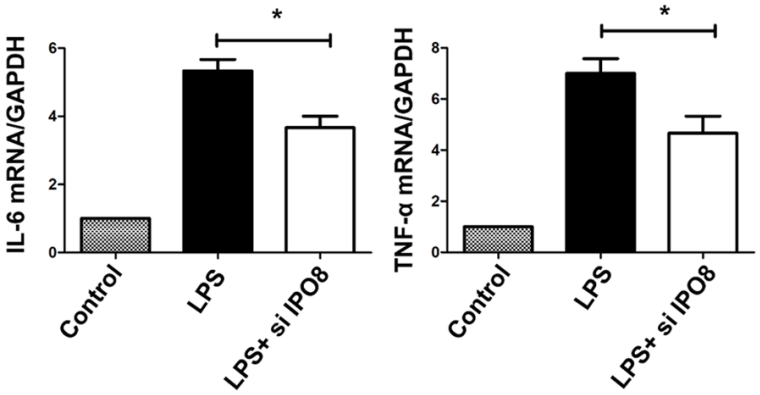

Figure 6. Quantitative PCR with small interfering (siRNA) knockdown for importin 8 showing decreased tumor necrosis factor (TNF)-α and interleukin (IL)-6 mRNA expression in mouse macrophage RAW264.7 cells stimulated with P. gingivalis lipopolysaccharide.

odontal inflammation (**Figure 3**). Positive expression was mainly observed in the cytoplasm (**Figure 4**).

LPS promoted IPO8 expression in macrophages

IPO8 expression in mouse macrophage RAW-264.7 cells was upregulated in cultures stimulated with 0.5 μg/mL *P. gingivalis* LPS compared with those without LPS stimulation, as detected by western blotting (**Figure 5**).

Knockdown of IPO8 by small interfering RNA

Knockdown of IPO8 alleviated the increase in TNF-α and IL-6 mRNA expression that was observed in mouse macrophage RAW264.7 cells stimulated with *P. gingivalis* LPS (**Figure 6**). Knockdown of IPO8 also decreased the expression of NF-κB pp 65 in the nucleus of mouse monocytic cells in vitro (**Figure 7A**). Silencing of IPO8 alleviated LPS-induced NF-κB activation (**Figure 7B**).

Discussion

IPO8 is specific for nucleocytoplasmic cargo in mammalian cells, functioning to mediate the transport between the cytoplasm and nucleus of macromolecules that contain nuclear import or export signals [7, 8]. As it is known to constantly shuttle between the nucleus and cytoplasm, we were not surprised to find IPO8 in both the nucleus and cytoplasm in our immunohistochemical experiments. We observed that tissues from patients with severe periodontitis had increased numbers of inflammatory infiltrating cells and IPO8-positive cells.

Periodontitis is an inflammatory disease caused by periodontal pathogens. Large numbers of inflammatory infiltrating cells are observed in human periodontitis [9]. 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 [10]. CD68 is particularly useful as a marker for the various cells of the macrophage lineage, which include monocytes, histiocytes, giant cells and osteoclasts [11-13]. An increase in CD68-positive cells was observed in parallel with the increase in inflammatory infiltration in this study. Infiltrating macrophages produce excess amounts of inflammatory cytokines and are involved in periodontal tissue destruction. Inflammatory infiltrating cells contribute to prolonged periodontal inflammation [10].

Our histologic results suggested that both IPO8 and macrophages play an important role in human periodontitis. To further examine this

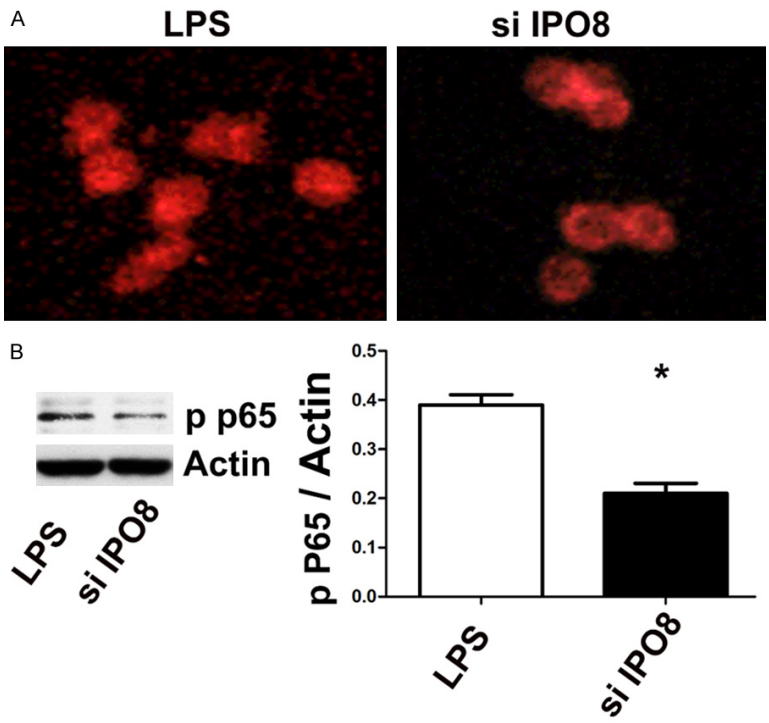

Figure 7. Immunofluorescence analyses showing decreased expression of nuclear factor (NF)-κB pp 65 in the nucleus of mouse monocytic cells in vitro with knockdown of importin 8 (IP08) (A). Silencing of IP08 alleviated lipopolysaccharide (LPS)-induced NF-κB activation (B).

role, we investigated IP08 expression in mouse macrophage RAW264.7 cells stimulated by *P. gingivalis* LPS. A rapid inflammatory response was produced in *P. gingivalis* LPS-stimulated RAW264.7 macrophages, which could release many different pro-inflammatory cytokines. TNF-α and IL-6 are two important proinflammatory mediators that are significantly increased during the progression of periodontitis, and are known to actively participate in periodontal tissue destruction [1]. We found that IP08 expression was also increased in mouse macrophage RAW264.7 cells. Knockdown of IP08 attenuated the LPS-induced increase in TNF-α and IL-6 gene expression in RAW264.7 cells. These results suggested that IP08 plays an important role in the LPS-induced inflammatory response. LPS stimulation increased the expression of IP08 and proinflammatory mediators TNF-α and IL-6 in vitro. The silencing of IP08 attenuated this increase in TNF-α and IL-6 in vitro.

NF-κB is an important transcription factor that regulates the expression of most proinflammatory cytokines. Once activated by LPS, phosphorylation of p65 is strongly enhanced, and is an obvious indicator of increased NF-κB activation [14]. In this study, the phosphorylation of p65 was reduced after silencing IP08 expression. Thus, the silencing of IP08 could inhibit the expression of inflammatory mediator and proinflammatory cytokine expression by downregulation of the NF-κB pathway.

In conclusion, our results indicate that IP08 plays a role in the progression of the inflammatory response in human periodontitis. Targeted inhibition of IOP8 expression can effectively alleviate periodontal inflammation.

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Disclosure of conflict of interest

None.

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References

[1] Yu X, Gong Z, Lin Q, Wang W, Liu S and Li S. Denervation effectively aggravates rat experimental periodontitis. J Periodontal Res 2017; 52: 1011-1020.

[2] Strom AC and Weis K. Importin-beta-like nuclear transport receptors. Genome Biol 2001; 2: REVIEWS3008.

[3] Wei Y, Li L, Wang D, Zhang CY and Zen K. Importin 8 regulates the transport of mature microRNAs into the cell nucleus. J Biol Chem 2014; 289: 10270-10275.

[4] Du Y, Yu XJ, Wang W, Zhao XY, Zhang LL, Sun YM, Wang X, Yu Q. Elevated expression of im-

portin 8 in inflamed human dental pulps. Int J Clin Exp Pathol 2017; 10: 7699-7706.
[5] Zhang T, Yu XJ, Liu S, Lv LL, Wang YZ, Li S. Effect of calcitonin-gene-related peptide on proliferation and osteogenesis in rat bone mesenchymal stem cells. Int J Clin Exp Pathol 2017; 10: 1074-1083.
[6] Yu X, Liu S, Wang W and Li S. Periodontal ligamentassociated protein1 delays rat periodontal bone defect repair by regulating osteogenic differentiation of bone marrow stromal cells and osteoclast activation. Int J Mol Med 2018; 41: 1110-1118.
[7] Weinmann L, Hock J, Ivacevic T, Ohrt T, Mutze J, Schwille P, Kremmer E, Benes V, Urlaub H and Meister G. Importin 8 is a gene silencing factor that targets argonaute proteins to distinct mRNAs. Cell 2009; 136: 496-507.
[8] Volpon L, Culjkovic-Kraljacic B, Osborne MJ, Ramteke A, Sun Q, Niesman A, Chook YM and Borden KL. Importin 8 mediates m7G cap-sensitive nuclear import of the eukaryotic translation initiation factor eIF4E. Proc Natl Acad Sci U S A 2016; 113: 5263-5268.
[9] Xue LD, Su LW, Xie JL, Du YM, Yu XJ. EMMPRIN-CypA contributes to the inflammatory processes in human periodontitis through infiltrating CD68+ inflammatory cells. Int J Clin Exp Pathol 2018; 11: 3828-3834.
[10] Yu X, Liu H, Liu S, Chen X, Zhao X, Du Y and Li S. Periodontal ligament-associated protein-1 gets involved in experimental periodontitis. J Periodontal Res 2019; 54: 180-189.
[11] Meng XM, Wang S, Huang XR, Yang C, Xiao J, Zhang Y, To KF, Nikolic-Paterson DJ and Lan HY. Inflammatory macrophages can transdifferentiate into myofibroblasts during renal fibrosis. Cell Death Dis 2016; 7: e2495.
[12] Radvar M, Shafaee H, Mohtasham N, Shiezadeh F and Zamanpour M. The effect of smoking on inflammatory cell infiltrate subtypes in gingival tissue of patients with chronic periodontitis. Electron Physician 2017; 9: 4961-4967.
[13] Xue L, Su L, Zhao L, Li J, Du Y and Yu X. Cyclophilin a increases CD68(+) cell infiltration in rat experimental periodontitis. J Mol Histol 2018; 49: 157-164.
[14] Martins GR, Gelaleti GB, Moschetta MG, Maschio-Signorini LB and Zuccari DA. Proinflammatory and anti-inflammatory cytokines mediated by NF-kappaB factor as prognostic markers in mammary tumors. Mediators Inflamm 2016; 2016: 9512743.