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Original Article

The expression levels of chemotaxis-related molecules CXC chemokine receptor 1, interleukin-8, and pro-platelet basic protein in gingival tissues

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Chemotaxis-related molecules

Abstract *Background/purpose:* Excessive host immune response is thought to be an important cause of periodontal tissue damage during periodontitis. The potent chemotaxis produced by locally released chemokines is the key signal to trigger this response. Here, we aimed to investigate the expression of CXC chemokine receptor 1 (CXCR1), and chemokines interleukin-8 (IL-8) and pro-platelet basic protein (PPBP) in human inflammatory gingival tissues compared with healthy tissues.
Materials and methods: A total of 54 human gingival tissues, 27 healthy and 27 inflammatory samples, were collected. Fifteen specimens of each group were employed for quantitative reverse transcription polymerase chain reaction to determine the mRNA levels of CXCR1, IL-8, and PPBP. Six samples of each group were used for Western blotting to investigate the protein expression of CXCR1 and for enzyme-linked immunosorbent assay to evaluate the protein levels of IL-8 and PPBP, respectively.
Results: The mRNA levels of chemokine receptor CXCR1, chemokine IL-8, and PPBP in inflammatory gingival tissues were significantly higher than those in healthy controls ($P < 0.05$). The protein levels of CXCR1, IL-8, and PPBP in inflammatory gingival tissues were also significantly higher than those in healthy gingival tissues ($P < 0.05$).

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Conclusion: When compared to healthy gingival tissues, the expression of CXCR1, IL-8, and PPBP in inflammatory gingival tissues is higher.

Introduction

Periodontitis is a chronic inflammatory disease that occurs in the supporting tissues of the periodontium.[1] The initiating factor of periodontitis is plaque. The interaction between the plaque biofilm and the host immune system determines the course and progression of periodontitis.[2] These pathogenic microorganisms can cause direct damage to periodontal tissues through their own metabolites as well as through the production of virulence factors.[3] However, with the increasing understanding of the pathogenesis of periodontitis, we are coming to realize that most of the tissue damage in periodontal diseases is due to the immune response generated by the host cells.[4] Via the local generation of chemokine when microbial attack or other pathological stimuli, inflammatory cells like neutrophils were attracted to the local inflammation area against bacteria.[5] However, an excessive buildup of neutrophils can result in the production of a number of proteases and oxidants that worsen inflammation and cause more tissue damage.[6] Hence, these chemokines are crucial in the pathological process.

Interleukin-8 (IL-8), also known as CXC motif chemokine ligand 8 (CXCL8), is considered to be one of the typical chemokines of the CXC family. It is mainly responsible for neutrophil recruitment and activation and has a powerful pro-inflammatory effect.[7] Many studies have revealed a close relationship between IL-8 and the development of periodontitis.[8] Patients with periodontitis were shown to have high levels of IL-8 in their saliva, gingival crevicular fluid, and gingival tissues.[9–11] CXC chemokine receptor 1 (CXCR1) is a high-affinity receptor for IL-8, which determines its irreplaceable position in periodontitis.[12] However, only two studies showed its expression level in human gingival tissues.[13,14] Pro-platelet basic protein (PPBP), is also a member of the CXC chemokine family. It has a similar structure to IL-8 and is also an inducer and activator of neutrophils.[6] PPBP has been found to be associated with a variety of inflammatory diseases, and some evidence supports the relationship between PPBP and host immune response.[15–17] However, there is still no report on the expression level of PPBP in human gingival tissues.

Recently, our group performed whole transcriptome sequencing. The results show neutrophil chemotaxis, chemokine activity, and CXCR binding were elevated in inflammatory gingival tissues according to the enrichment analysis of the C5 GO genomic dataset.[18] In addition, we identified 10 hub genes from the protein-protein interaction network using topological analysis. And most of the hub genes were associated with chemokines and chemokine receptors. We found that CXCR1 and PPBP were also included.[18] As CXCR1 and PPBP are important members of chemotaxis-related molecules, and play an important role in the induction and activation of neutrophils together with IL-8, it is of interest how their expression in gingival tissues.

Hence, the objective of this research is to investigate the expression of the chemokine receptor CXCR1, and chemokines IL-8 and PPBP in healthy and inflammatory gingival tissues.

Materials and methods

Collection of gingival tissue samples

The gingival samples were collected during periodontal surgery from patients (aged 18 to 65) who visited the Peking University Stomatology Hospital. All the participants are systemically healthy. In periodontitis patients, teeth with probing depth $\geq$6 mm and bleeding index $\geq$2 and with intrabony defect, based on clinical and radiographic examination, were selected as the sample teeth. The inflammatory gingival tissues were collected during flap surgery. Gingival samples as control group were obtained from teeth with probing depth $\leq$3 mm and bleeding index $\leq$2 during crown lengthening surgery. The gathered gingival tissues were cleaned with 0.9% saline and put right away in sterile cryogenic vials. After that, they were submerged in liquid nitrogen, and moved as soon as possible to a −80 °C refrigerator for storage. The ethical committee of the Peking University Hospital of Stomatology gave the study approval with the approval code PKUSSIRB-202384004.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The tissues were lysed using TissueLyser II (QIAGEN, Dusseldorf, Germany). And the total RNAs were extracted using the Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. The ABScript II cDNA First Strand Synthesis Kit (Abclonal, Wuhan, China) was utilized to reverse transcribe total RNA. Incubation at 37 °C for 2min was followed by 55 °C for 15min, 85 °C for 5min to inactivate the enzyme, and keeping warm at 4 °C in the PCR. QRT-PCR was performed as previously described using qPCR SYBR Green Master Mix (Abclonal). The following is a list of the primer sequences utilized in this study: GAPDH (forward: 5′-TCATTTCCTGGTATGACAACGA-3'; backward: 5′- GTCTTACTCCTTGGAGGCC-3′), CXCR1 (forward: 5′- AACCTAGCCATCGCCGACCTAC-3′; backward: 5′- CAGCAGCAGGATACCACTGAAGAAG-3′), IL-8 (forward: 5′- GCTGCTCAAGGCTGGTCCATG-3′; backward: 5′- CATCGTAGCTCTTGAGTGTCACAGG-3′),

PPBP (forward: 5′- TGGGCTTCAGACTCAGACCTACATC-3′; backward: 5′- TGGGTCCATGCCATCAGATTTTCC-3′).

Western blot analysis

The gingival tissues were lysed using RIPA buffer (Solarbio, Beijing, China) for 15min and cleavage by the TissueLyser II (QIAGEN). Using a BCA determination kit (Thermo, Waltham, MA, USA), protein concentrations were evaluated. The protein samples were loaded at 40μg/well onto 10% Express Cast PAGE Gel Preparation kit (New Cell & Molecular Biotech, Jiangsu, China), and then transferred to polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). At room temperature for 1.5 h, 5% skimmed milk was used to block the membrane. The membrane was incubated with *anti*-CXCR1 (Abclonal) and *anti*-β-Actin (Abclonal) using the concentration of 1:1000 at 4 °C, and then incubated with a diluted 1:10000 concentration of Horseradish peroxidase-conjugated secondary antibody (ZSGB-Bio, Beijing, China) for 1 h after being rinsed three times with TBST. After titration of ECL solution (Huaxingbio, Beijing, China), the membrane was analyzed using Touch Imager Contact Chemiluminescence Imaging Systems (e-BLOT, Shanghai, China).

Enzyme linked immunosorbent assay (ELISA)

The TissueLyser II (QIAGEN) was used to cleavage the tissues after they had been collected, weighed, and the appropriate volume of PBS (Servicebio, Beijing, China) had been added based on the mass. The concentration of IL-8 and PPBP were evaluated by ELISA method according to the manufacturer's instructions (Meimian, Jiangsu, China).

Statistical analysis

Statistical analysis was performed using SPSS software (Version 27.0) (IBM, Armonk, NY, USA). Differences between normally distributed values were analyzed by a two-tailed *t*-test. If the values were nonnormally distributed, Mann-Whitney non-parametric test were performed. Statistical significance was set at *P*-value <0.05.

Results

The mRNA levels of CXCR1, IL-8 and PPBP in healthy and inflammatory gingival tissues

The mRNA levels of CXCR1, IL-8 and PPBP were investigated in 15 healthy gingival tissues and 15 inflammatory gingival tissues using qRT-PCR. The findings revealed that, the mRNA levels of CXCR1, IL-8 and PPBP were all increased in gingival inflammatory tissues when compared to the healthy controls (Fig. 1).

The protein levels of CXCR1, IL-8 and PPBP in healthy and inflammatory gingival tissues

In order to identify the protein expression of CXCR1, IL-8 and PPBP, six healthy gingival samples and six inflammatory gingival samples were employed for Western blot analysis or ELISA, respectively. In contrast to the healthy controls, the inflammatory gingival tissues had higher protein levels of CXCR1, IL-8 and PPBP (Fig. 2).

Discussion

Our study investigated the expression of the chemokine PPBP in human healthy and inflammatory gingival tissues for the first time. The results showed that both the mRNA and protein of PPBP were significantly higher in inflammatory gingival tissues in comparison to healthy controls. High expression of PPBP was found in the plasma or serum of various inflammatory, allergy, and cardiovascular disorders,[15–17,19] which raises the possibility that PPBP contributes to the host immune response. However, the relationship between PPBP and periodontitis is still unclear. Steinberg et al. assayed for β-thromboglobulin levels in gingival crevicular fluid, and measured the gingival index to evaluating the degree of gingival inflammation.[20] They reported that β-thromboglobulin was enhanced expression at gingival crevicular fluid of periodontitis sites and concluded that β-thromboglobulin is closely linked to gingival inflammation.[20] β-platelet globulin is known as a derivative of PPBP.[21] The result suggests that PPBP may be related to

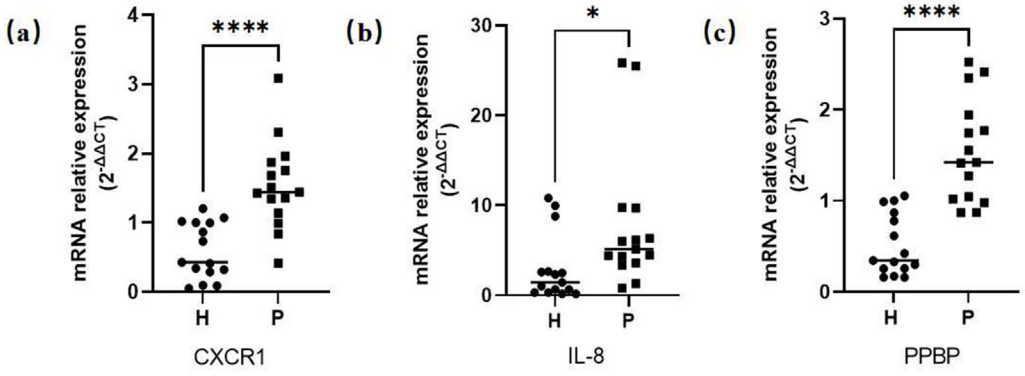

Figure 1 The mRNA levels of CXCR1, IL-8 and PPBP in inflammatory gingival tissues and healthy tissues. (a–c) The relative expression levels of CXCR1, IL-8 and PPBP were expressed by $2^{-\Delta\Delta CT}$. H: healthy gingival tissues (n = 15), P: inflammatory gingival tissues (n = 15). *: $P < 0.05$, ****: $P < 0.0001$.

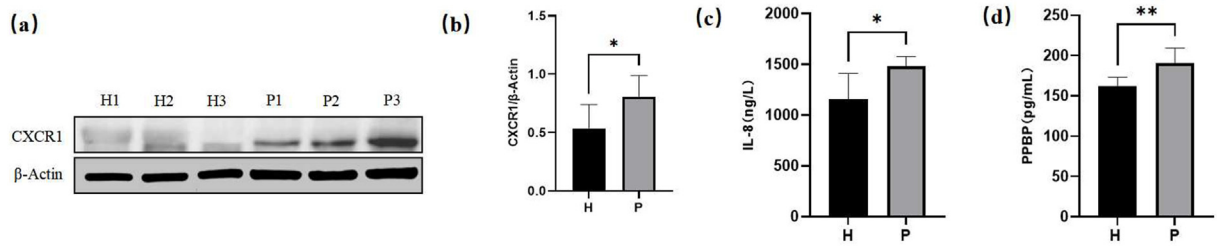

Figure 2 The protein levels of CXCR1, IL-8 and PPBP in inflammatory gingival tissues and healthy tissues. (a) H1–H3: protein band of healthy gingival tissues, P1–P3: protein band of inflammatory gingival tissues. (b) Western blot analysis of CXCR1. Perform grayscale analysis on six healthy gingival tissues and six inflammatory gingival tissues, with CXCR1 grayscale values/β- Actin grayscale value as the vertical axis. (c-d) ELISA results of IL-8 and PPBP. H: healthy gingival tissues (n = 6), P: inflammatory gingival tissues (n = 6). *: $P < 0.05$, **: $P < 0.01$.

periodontitis. To our knowledge, there still no reports about the expression level of PPBP in human gingival tissues in the previous literature. PPBP is a member of the chemokine family and involved in the induction and activation of neutrophils.[22] Prior studies found that PPBP increases neutrophil transendothelial migration and specific adherence to endothelial cells.[23] Neutrophils is known as the first line of periodontal local host defense. However, we all know that excessive host immune response is the main source of periodontal tissue damage. Therefore, the high expression of PBPP in inflammatory gingival tissues may involve the pathogenesis of periodontal disease.

There is little direct evidence of PPBP's relationship with periodontal diseases. A previous study suggests that platelet factor 4/PPBP/CXC motif chemokine ligand 5 gene cluster is a promising candidate gene related to human periodontitis.[24] Miyauchi et al. used C57BL/6. KOR-*ApoE*^{shl} mice to analyze the effect of oral infection with *Porphyromonas gingivalis* on the expression levels of multiple cytokines in the serum, which is a useful model for investigating the effect of the periodontitis-induced systemic inflammatory response on atherogenesis. They reported that PPBP is specifically elevated in the serum of *Porphyromonas gingivalis*-infected mice compared with sham-infected mice.[25] PPBP has been found in the plasma of people with diabetes mellitus,[15] coronary artery disease,[19] and the serum of people with rheumatoid arthriti.[17] It is believed to be a possible biomarker for these diseases. These diseases were reported to be related with periodontitis, and the two-way relationship between diabetes and periodontitis in particular is well known.[26–28] However, the processes underlying the connection between diabetes and periodontitis are not entirely understood, and may involve features of cytokine biology and neutrophil activity.[29] PPBP is thought to be a potent neutrophil inducer and activator.[30] In the current investigation, inflammatory gingival tissues showed a considerable upregulation of PPBP. Although it was shown that people with type II diabetes had high levels of this chemokine in their plasma,[15] it is unknown if PPBP bridges the gap between periodontitis and diabetes.

This study also revealed for the first time that the chemokine receptor CXCR1 was substantially expressed at both the mRNA and protein levels in inflammatory gingival tissues. CXCR1 expression levels in inflammatory gingival tissues have also been documented in two earlier investigations.[13,14] But just the mRNA or protein level was investigated in these two investigations, respectively. Noda et al. obtained 24 gingival tissues from 22 individuals who had chronic periodontitis after periodontal surgery.[13] The mRNA levels of various chemokines in inflammatory gingival tissues were measured using qRT-PCR. And CXCR1&2 were found to be widely expressed in inflammatory tissues of patients with periodontitis compared to other chemokine receptors, such as CXC chemokine receptor 3, CC chemokine receptor 1, and CC chemokine receptor 2.[13] Sfakianakis et al. used immunohistochemistry to detect CXCR1 protein of eight inflammatory gingival tissues and four healthy controls, and discovered that CXCR1 protein was more broadly expressed in inflammatory tissues than in the healthy ones.[14] In this study, we evaluated the mRNA levels of CXCR1 in 30 gingival samples and the protein levels in 12 gingival tissues, and discovered that CXCR1 expression was higher in inflammatory tissues. CXCR1 is a high-affinity receptor for IL-8 and internalized by binding to ligands and phosphorylating, which in turn activates a variety of intracellular pathways, including mitogen-activated protein kinase and protein kinase C, and ultimately nuclear factor-κB, hypoxia-inducible factor-1, and signal transduction.[31–34] CXCR1 mediates neutrophil activation and adherence,[32] which establishes its undeniable significance for tissue damage in periodontal immune response.

Additionally, the elevation of IL-8 in inflammatory gingival tissues has been demonstrated in both our study and earlier research.[35,36] Min et al. evaluated the levels of IL-8, interleukin-6, and TNF-α protein expression in gingival tissues from 19 individuals with periodontitis by ELISA. The protein expression levels of TNF-α were consistently high and fluctuated in a small range. In contrast, the protein levels of IL-6 and IL-8 showed significant fluctuations, with IL-8 showing the highest levels among the three.[35] The wide range of fluctuations of IL-8 is consistent with the results of the present study. Previous studies have found that gingival fibroblasts from patients with periodontitis secrete higher levels of IL-8 compared to healthy controls.[11] In addition, numerous investigations have revealed greater amounts of IL-8 in the gingival crevicular fluid of periodontitis sites and in the saliva of periodontitis patients,[9,37] indicating that inflammatory sites may have a more potent chemotactic effect through IL-8 overexpression. The higher levels of IL-8

found in specific gingival tissues of healthy controls in this study may be attributable to some pathological stimuli that, despite the absence of significant periodontal destruction, may lead to upregulation of IL-8 in these sites and which occur before the presence of clinically observable lesions.[38]

CXCR1, IL-8 and PPBP are chemotaxis-related molecules, which are closely related to the development of periodontitis. Previous research has demonstrated that periodontal pathogen-induced inflammatory and immune responses can, on the one hand, shield the host from infection. However, on the other hand, a persistent, chronic host response may change the protective function and harm periodontal tissues. Chemokines present in gingival tissue and gingival sulcus fluid are believed to play a significant role in the immunopathogenesis of periodontal diseases. The development of periodontal diseases appears to be linked to the infiltration of inflammatory cells into deeper periodontal tissue.[39] Therefore, it is thought that research into these chemokines and receptors is a crucial tool for understanding the pathophysiology of periodontal disorders.[8,40] This study not only provides more sufficient evidence for the high expression of CXCR1 in inflammatory gingival tissues, but also identifies PPBP as a potential novel biomarker for periodontitis.

Declaration of competing interest

The authors have no conflicts of interest in this study.

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