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The impact of smoking on levels of chronic periodontitis-associated biomarkers

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ABSTRACT

Objective: To investigate the effect of smoking on the expression levels of matrix metalloproteinase (MMP)-1, MMP-9, tissue inhibitor of metalloproteinase-1 (TIMP-1), and the concentrations of TNF-α and IL-10 in patients with chronic periodontitis (ChP).

Methods: This is an ex-vivo study. Our study consisted of 78 cases, all of which were diagnosed with ChP and were selected according to the inclusion and exclusion criteria. Among these 78 cases, 38 patients were classified into the smoking group (S-ChP group), and 40 patients in the non-smoking group (NS-ChP group). The clinical periodontal parameters of all patients were recorded, including the plaque index (PLI), probing depth (PD), loss of attachment (LA) and sulcus bleeding index (SBI). Serum was collected from forearm blood to establish a *Porphyromonas gingivalis* (*Pg*) internalizing KB cell model. Enzyme-linked immunosorbent assay (ELISA) was used to determine the concentrations of MMP-1, MMP-9 and TIMP-1 in the KB cell lysis solution as well as IL-10 and TNF-α in the gingival crevicular fluid (GCF).

Results: Fewer *Pg* internalizing KB cell colonies were observed in the NS-ChP group than in the S-ChP group ($P < 0.01$). When 400 μL serum was added, there were remarkable differences in the concentrations of MMP-1 and TIMP-1 secreted from the KB cells between the S-ChP and NS-ChP groups (MMP-1: $t = -21.71$, $P < 0.01$; TIMP-1: $t = 64.35$, $P < 0.001$). Additionally, when 800 μL serum was added, there were significant differences in the concentrations of MMP-1, MMP-9 and TIMP-1 in the KB cells between the S-ChP and NS-ChP groups (MMP-1: $t = -81.89$, $P < 0.001$; MMP-9: $t = -15.67$, $P < 0.001$; TIMP-1: $t = 109.4$, $P < 0.001$). The TNF-α levels were higher, but the IL-10 levels were lower in the GCF from the ChP patients in the S-ChP group than those in the NS-ChP group (both $P < 0.001$).

Conclusion: The serum of S-ChP patients can enhance the concentrations of MMP-1 and MMP-9, but reduce TIMP-1 secreted from *Pg* internalizing KB cells. However, the concentration of TNF-α was increased and IL-10 was decreased. Abnormal concentrations of ChP-associated biomarkers may be conducive to the development and progression of ChP.

1. Introduction

Periodontitis, a chronic inflammatory disease, is characterized by loss of periodontal ligament and alveolar bone, and can lead to tooth loss and affect individual's oral health-related quality of life (De Pablo et al., 2009; Durham et al., 2013). It is estimated that severe periodontitis affects 5–20% of any population, and mild/moderate periodontitis is a problem of majority of adults (Kassebaum et al., 2014; Dye, 2012; Petersen and Ogawa, 2012). Periodontitis is more common among men than women and is more prevalent in developing areas than in developed areas, and the occurrence of this disease shows an upward with aging (Ababneh et al., 2012; Darveau, 2010). Chronic periodontitis (ChP) can be developed by modifiable risk factors, including cigarette smoking, alcohol consumption, poor diet, and psychological stress and depression (Reynolds, 2014; Filoche et al., 2010). It has been reported that smoking is strongly involved in the initiation and progression of periodontitis disease and that smokers were found altered amount of gingival crevicular fluid (GCF) and its components (Mokeem et al., 2014; Kubota et al., 2011). Smoking is also reported to have a potential negative effect on the immune defense system of the periodontal host, which inhibits neutrophil and macrophage function and affects defense against bacteria (Gomes et al., 2015; Fan et al., 2015).

Matrix metalloproteinases (MMPs) are zinc- and calcium-dependent proteolytic enzymes that are responsible for the degradation of all extracellular matrix proteins and basement membrane components

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(Marcaccini et al., 2010). MMPs can be produced by inflammatory cells caused by bacterial infection in response to cytokines secreted from these cells in periodontal patients; thus, their presence, quantities, and activities are recognized to be very important for the characterization of various states of periodontitis and the determination of active destruction (Kim et al., 2013). Endogenous tissue inhibitors, especially tissue inhibitors of metalloproteinases (TIMPs), are significant factors in the control of MMPs (Mouzakiti et al., 2012). An imbalance between MMPs and TIMPs is found in pathological conditions, including periodontitis and correlates with tissue destruction (Verstappen and Von den Hoff, 2006; Kubota et al., 2008). Tumor necrosis factor alpha (TNF-α), as a pro-inflammatory cytokine, can lead to propagation of inflammation and contribute to release of a high level of inflammatory mediators, which result in destruction of tooth-supporting periodontal structures (Passoja et al., 2010; Yousefimanesh et al., 2013). As a vital anti-inflammatory cytokine that impedes inflammatory responses, IL-10 is also implicated in ChP (Zhang et al., 2014). *Porphyromonas gingivalis* (*Pg*) is a predominant periodontal pathogen that expresses multiple potential virulence factors involved in the pathogenesis of periodontitis (Amano, 2007). The virulence factors enable *Pg* to invade the periodontal tissue and subsequently spread into the systemic circulation (Blasco-Baque et al., 2016). *Pg* can also invade host cells, including epithelial cells (Amano et al., 2014). Gingival epithelial cells are spontaneously exposed to bacterial attacks and function in preventing bacteria invasion into deeper tissues (Amano, 2007). In the present study, we detected the levels of MMP-1, MMP-9, TIMP-1 in the supernatants of *Pg* internalizing KB cells and the levels of TNF-α and IL-10 in the GCF, in order to explore the effect of smoking on ChP.

2. Materials and methods

2.1. Subjects

A total of 78 patients diagnosed with ChP were recruited into our study from the First Affiliated Hospital of Jinzhou Medical University between September 2012 and March 2015. The ChP was diagnosed in accordance with the recommended standards from the Center for Disease Control and Prevention, the American Academy of Periodontology, on the condition of (1) presence of ≥2 interproximal sites with ≥3 mm clinical attachment loss, not on the same tooth, and (2) presence of ≥2 interproximal sites with ≥4 mm probing depth (PD) occurring at two or more different teeth, or presence of ≥5 mm PD of a site (Armitage, 1999). According to the standard of WHO definition of the smoking population, patients who smoked >10 cigarettes daily for longer than 2 years were considered smoking patients with ChP (S-ChP group). The patients who quit smoking for >10 years or had no smoking habits were considered non-smoking patients with ChP (NS-ChP group). Our study consisted of 38 cases of S-ChP and 40 cases of NS-ChP. The exclusion criteria for patient enrollment and experimental teeth were as follows: (1) patients with systemic disease; (2) patients taking oral antibiotics, analgesics or hormonal drugs in the past three months, and those who had received periodontal treatment for nearly half of the year; (3) patients with <20 remaining teeth, and experimental teeth with periapical disease (based on clinical symptoms, X-ray examination and diagnostic results) and occlusal trauma (based on functional mobility, occlusion widened periodontal ligament and other local incentives diagnosis). In addition, periodontal clinical parameters were obtained from each patient with ChP, including the plaque index (PLI), PD, loss of attachment (LA) and sulcus bleeding index (SBI). Two diagonal quadrants were randomly selected where a tooth with the highest PD was used as the experimental molar tooth and the area with the deepest PD was the GCF sample site. This study was approved by the Ethics Committee of the First Affiliated Hospital of Jinzhou Medical University, and the subjects' written consent was obtained according to the current Declaration of Helsinki.

2.2. Clinical parameter measurement and sample collection

The periodontal clinical indices of the ChP patients were recorded. The PLI was determined and divided into four grades on the basis of the Silness & Loe PLI methods. The scores were as follows: grade 0, no plaque in the gingival margin area; grade 1, thin plaque in the gingival margin of the tooth surface that was not easy to see with the naked eye but could be scraped using the tip side of a probe; grade 2, visible medium plaque in the gingival margin or interproximal surface; grade 3, a large amount of soft scale in the gingival margin and the adjacent surface or gingival sulcus. The PD was the distance from the gingival margin to the bottom of periodontal pocket or the bottom of gingival sulcus. The LA was the difference between the PD and the distance from the enamelo-cemental junction to the gingival margin; if gingival recession was present, the PD and the gingival recession distance were summed. The SBI was divided into six grades according to the gingival bleeding degree by Florida periodontal probing. The grades were as follows: grade 0, patients with healthy gingival, no inflammation or bleeding symptoms; grade 1, patients with gingival color changed with inflammation but no bleeding from probing; grade 2, punctate hemorrhage after probing; grade 3, probe hemorrhage diffusion along the gingival margin; grade 4, full bleeding and gingival fluid overflow; grade 5, spontaneous bleeding.

Two diagonal quadrants were randomly selected in the mouth of each study subject. The molars or premolars were selected as experimental teeth on the condition that: no filling body or caries were present at the tooth neck; PD > 3 mm; LA more than or equal to 1 mm; and varying degrees of destruction or resorption of alveolar bone evident by X-ray. The GCF samples were taken from the deepest site of the PD. Filter paper strips (2 mm × 10 mm) were placed at the bottom of the periodontal pocket and kept in place for 30 s to adsorb and collect the GCF. Four sites were selected, including the buccal side, far from the buccal side, the lingual side and far from the lingual side. The four filter paper strips were placed into the same EP tube, followed by sealing, labelling, weighing and recording, and then reserved at 80 °C in a low-temperature refrigerator for further use. The reserved GCF sample was taken out from the refrigerator. After back to room temperature, the sample was shocked, eluted for 1 h and centrifuged (13,000 r/min) for 10 min at 4 °C. The supernatant was collected in clean EP tubes (each 50 μL). All the above steps were completed by a highly-qualified and professionally trained physician from our hospital.

2.3. Bacterial culture

Venous blood (5 mL) was collected from the forearms of all of the study subjects and centrifuged to obtain serum samples, which were then reserved at −80 °C. After PgATCC33277 recovery at room temperature, it was inoculated into brain heart infusion agar (BHIA) medium containing 1% hemoglobin chloride, 5% sterile defibrinated sheep blood, and 0.1% vitamin K1 at 37 °C in an anaerobic environment for 5–7 days. Gram staining and biochemical identification were performed to confirm the purity of PgATCC33277, which was then cultured at 4 °C for 24 h. After 10 min of centrifugation at 5000 r/min, the bacteria were collected. The bacteria were washed with PBS and suspended in Dulbecco's modified Eagle's medium (DMEM, Gibco, Life Technologies, Darmstadt, Germany) without an antibiotic. Then, a UV spectrophotometer was used to measure the absorbance at 600 nm, and the bacterial suspension was adjusted to 1×10^8 CFU/mL. Fifty samples were obtained randomly after the enrichment of the bacterial liquid extraction, and Gram staining and polymerase chain reaction (PCR) were used to identify *Pg* using the upstream primer 5′-TGTAGATGACTGATGGTGAAAACC-3′ and the downstream primer 5′-ACGTCATCCACACCTTCCTC-3′.

2.4. Cell culture

The conventional KB cell line ATCCCCL17 was thawed from cryopreservation, inoculated in low-glucose DMEM and incubated in a