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Protease-activated receptors expression in gingiva in periodontal health and disease

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ABSTRACT

Objective: Protease-activated receptors (PARs) are a unique class of receptors which are implicated in mediating inflammation, pain and other functions. The aim of this study was to elucidate the role of PARs in the pathogenesis of chronic periodontitis by differential expression analysis of PARs in the gingival tissues of chronic periodontitis patients compared with those of healthy control individuals.

Design: Gingival tissue specimens were collected from chronic periodontitis patients ($n = 20$) and control individuals ($n = 20$). The expression of PAR-1, -2, -3 and -4 was determined in these tissues by immunohistochemistry and differential expression between the two groups was investigated by quantitative real-time reverse transcription-polymerase chain reaction analysis.

Results: PAR-1, -2, -3 and -4 were expressed in all gingival tissues. A significant overexpression of PAR-3 was detected in chronic periodontitis-affected tissues compared to healthy gingival tissues. However, expression of PAR-2 was decreased in periodontal lesions.

Conclusions: Our study shows that PAR-1, -2, -3 and -4 are expressed in both healthy and inflamed gingival tissues. Furthermore, PAR-2 and PAR-3 may contribute to the inflammatory responses associated with chronic periodontitis.

1. Introduction

Chronic periodontitis, which is one of the most common oral diseases, is characterized by gingival inflammation and causes periodontal tissue destruction, loss of alveolar bone, and eventually, tooth loss. *Porphyromonas gingivalis* (*P. gingivalis*), which is the causative pathogen of chronic periodontitis,[1,2] possesses a number of putative virulence factors, namely lipopolysaccharide, capsule, gingipains and fimbriae, all of which stimulate host cells to release inflammatory mediators and promote this infectious disease.[3] Proteases produced by *P. gingivalis* have been shown to act as important pathogenic agents. Recently, it has been shown that proteases are recognized by cells via the family of protease-activated receptors (PARs), which are involved in inflammatory processes in several tissues.

Members of PAR family are G protein-coupled, seven-transmembrane-domain receptors that mediate various

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cellular responses to proteases such as thrombin, trypsin, and mast cell tryptase.[4,5] Four PAR family members (PAR-1, -2, -3 and -4) have been identified so far. The activation of PARs occurs by a unique mechanism that involves specific proteolytic cleavage of the N-terminal extracellular sequence by a protease. This cleavage unmasks a new N-terminal sequence, which acts as a tethered ligand that binds to the receptor to initiate multiple signalling cascades.[6–8] Although they share the same mechanism of action, it has been shown that different PARs are characterized by different distributions and biological actions and can be activated by different proteases.[9] It has been demonstrated that PAR-2 is activated by trypsin and human mast cell tryptase, whereas PAR-1, PAR-3, and PAR-4 are considered thrombin receptors.[10,11] However, PAR-4 is also activated by trypsin, cathepsin G, activated factor X of the coagulation cascade, and trypsin IV.[12–14] As PARs are expressed in a wide variety of cell types, it has recently been suggested that they play important roles in physiological processes such as growth, development, imflammation, tissue repair and pain.

PARs were found to be expressed in the epithelium of gingiva and have been implicated in the pathogenesis of periodontal disease in an animal model.[15–17] PARs was found in human gastric mucosa and human gingival epithelium by immunohistochemistry.[17,18] However, the precise roles of PARs in gingival tissues and the importance of specific PARs in the pathogenesis of periodontitis remain to be elucidated. In the present study, we used immunohistochemistry and quantitative real-time reverse transcription polymerase chain reaction (QRT-PCR) analysis to investigate the differential expression of PARs in the gingival tissues of chronic periodontitis patients compared with healthy control individuals in order to further elucidate the role of PARs in the pathogenesis of chronic periodontitis.

2. Materials and methods

2.1. Collection of samples

Gingival tissue specimens ($n = 40$) were obtained from the Department of Periodontology and the Department of Oral and Maxillofacial Surgery, The Affiliated Stomatology Hospital, School of Medicine, Zhejiang University (Hangzhou, China). All participants provided case histories and submitted to clinical, periodontal and radiographic examination. All teeth were scored by a single calibrated investigator (Dr. Hu) for probing depth and clinical attachment level-at six sites per tooth. Chronic periodontitis was defined as having ≥5 teeth with periodontal sites with probing depth (PD) ≥5 mm, clinical attachment level (CAL) ≥3 mm, and extensive bone loss determined radiographically. The patients were systemically healthy with no evidence of known systemic modifiers of periodontal disease (types I and II diabetes mellitus, osteoporosis and medication known to influence periodontal tissues). Exclusion criteria included those patients who had taken systemic antibiotics, anti-inflammatory agents, hormonal or other assisted drug therapy in the 6 months prior to the study, or those who had received previous periodontal therapy in the last 2 years. Smokers were not specifically excluded. Inflamed gingival tissues (PD ≥5, CAL ≥3 mm, extensive radiographic bone loss and sulcular bleeding on probing) were obtained during periodontal surgery or tooth extraction. Healthy gingival tissues were obtained during extraction of teeth for orthodontic purposes or due to impaction. Samples of gingival tissues were obtained from 20 chronic periodontitis patients (27 males and 33 females, aged 30–70 years old) and 20 healthy control individuals (10 males and 10 females, aged 26–40 years). We used 6 chronic periodontitis samples and 8 healthy control samples for QRT-PCR and 14 chronic periodontitis samples and 12 control samples for immunohistochemical staining. Written informed consent was obtained from all individuals participating in this study. The protocol and consent forms for the study were evaluated and approved by the Ethics Committee of Zhejiang University. Before collection of gingival tissue samples, Subgingival plaque samples were obtained by inserting sterile endodontic paper points into the pre-selected subgingival sites for 10 s. Paper points were placed in sterile Dnase and Rnase-free microcentrifuge tubes, and stored at −80 °C until processed.

2.2. Immunohistochemistry

Gingival biopsies were fixed in 10% neutral formalin, embedded in paraffin, and 3- to 4-μm sections were prepared and collected on poly-L-lysine coated slides. The slides were deparaffinized and dehydrated with xylene, ethanol (100%, 95%, 85%, and 75%), and distilled water. The samples were incubated overnight at 4 °C with mouse monoclonal antibodies against PAR-1, -2 or rabbit polyclonal antibodies against PAR-3, -4 (diluted 1:100) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Isotype-matched antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were used as negative controls. After washing with phosphate buffered saline (PBS), the sections were incubated for 20 min at room temperature with biotinylated anti-mouse or anti-rabbit immunoglobulin (diluted 1:200) (Jackson ImmunoReseach, Laboratories, Inc., PA, USA) and washed with PBS to remove unreacted antibodies. The sections were then treated with SABC (streptavidin–biotin complex) (Boster Biological Technology, Ltd., Wuhan, China) for 20 min at room temperature, washed and reacted with DAB (3,3-diaminobenzidine tetrahydrochrolide) (streptavidin–biotin complex) (Boster Biological technology, Ltd., Wuhan, China) to develop colour. The sections were counterstained with haematoxylin and mounted with glycerol.

2.3. Quantitative RT-PCR analysis

Tissue samples were submerged in ribonucleic acid (RNA)-stabilizing reagent (Omega Bio-Tek, Inc., Norcross, GA, USA) and transported at room temperature to be processed for RNA extraction. Total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA, USA) and reverse-transcribed using SuperScript RT-PCR kit (Takara, Tokyo, Japan). Quantitative RT-PCR was performed using the Applied Biosystems 7500 PCR machine and SYBR Premix kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. PCRs were carried out in 96-well plates in a total volume of 20 μl, including 1 μl of cDNA and 0.8 μl of primers (10 μM) (Table 1). Sample expression was normalized against that of the