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Expression of matrix metalloproteinases-2, -9 and reversion-inducing cysteine-rich protein with Kazal motifs in gingiva in periodontal health and disease

Nian Liu, Yingguang Cao, Guangxun Zhu*

Department of Stomatology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China

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Background and objective: Periodontitis is characterized by pathological destruction of extracellular matrix (ECM) of periodontal tissues. Matrix metalloproteinases (MMPs) promote the occurrence and development of periodontitis by degrading almost all proteins of ECM. RECK (reversion-inducing-cysteine-rich protein with kazal motifs), a novel membrane-anchored inhibitor of MMPs, could regulate the expression of MMP-2 and MMP-9 at post-transcriptional level. The study was to investigate the expression of RECK in healthy and diseased human gingival tissues and to correlate it with the production of MMP-2 and MMP-9.
Material and methods: Gingival biopsies were collected from chronic periodontitis patients and periodontally healthy control individuals. The protein and mRNA of RECK, MMP-2 and MMP-9 was determined by immunohistochemistry and semi-quantitative polymerase chain reaction analysis.
Results: The expression of RECK protein was mainly confined to the gingival epithelium in inflamed and non-inflamed gingival tissues. Expression of RECK was significantly lower in tissues from chronic periodontitis patients, while the positive expression levels of MMP-2 and MMP-9 in periodontitis specimens were significantly higher. RECK protein expression was negatively correlated to the expressions of MMP-2 and MMP-9 in periodontitis. Moreover, RECK mRNA was significanly lower in diseased gingiva than in healthy samples($P < 0.05$), while MMP-2 and MMP-9 mRNAs were observed overexpressed in periodontal lesions, with no significant correlation between RECK and MMP-2/MMP-9 mRNA shown in periodontally diseased group.
Conclusion: The expression of RECK in human healthy and diseased gingiva may contribute to periodontal physiological and pathological processes; low RECK expression may be associated with the enhanced MMP-2 and MMP-9 production in inflamed gingiva.

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1. Introduction

Periodontitis, representing the second most prevalent oral diseases, is a biofilm-induced chronic destructive periodontal inflammation that eventually leads to the loosening and loss of teeth. Generated from the imbalance between the subgingival microorganisms and the over-aggressive host defense (Sanz, van Winkelhoff, & Working Group 1 of Seventh European Workshop on Periodontology, 2011), the pathological process of periodontitis is characterized by the destruction of tooth-supporting structures that results from the degradation and remodelling of extracellular matrix(ECM), which is principally mediated by the orchestrated stimulation of a large group of proteolytic host enzymes (Reynolds and Meikle, 1997). Most notable in this group are matrix metalloproteinases(MMPs), a family of zinc-dependent peptidases that are capable of degrading most components of ECM, being considered to be closely related to the periodontium breakdown (Coussens, Fingleton, & Matrisian, 2002).

The basal membrane (BM) and extracellular matrix (ECM) are the first and foremost barrier to protect the periodontal tissues from inflammation. In the periodontal inflammation, host cells produce and release proinflammatory mediators as well as other reactive proteinases under the stimulation of bacteria, then the resident cells in the periodontium such as gingival fibroblasts, monocyte/macrophages and epithelial cells are activated to secrete cytokines, cysteine proteinases and MMPs in response (Sorsa et al., 2006). The periodontal ECM mainly consists of collagens(type I–IV)

* Corresponding author at: Department of Stomatology, Tongji Hospital, No.1095 Jiefang Road, Wuhan, Hubei, 430030, China.
E-mail address: zhuguangxun@163.com (G. Zhu).

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and non-collagen proteins such as elastin, gelatin, fibronectin and laminin. MMPs are capable of degrading almost all constituents of the matrix, among which MMP-2 and MMP-9 are classified as gelatinases that are responsible for the gelatin-binding properties (Bjorklund and Koivunen, 2005). 92 kDa Gelatinase B(MMP-9) is expressed by a variety of cells such as keratinocytes, osteoclasts, eosinophils, neutrophils and macrophages, while 72 kDa gelatinase A(MMP-2) is more widespread in fibroblasts, endotheliocytes, macrophages and malignant tumor cells (Sorsa et al., 2006). MMP-2 and MMP-9 expression and activity have been detected being increased in various kinds of oral inflammatory diseases such as periodontitis, peri-implantitis and pericoronitis (Ma et al., 2003; Westerlund et al., 1996; Beklen, Laine, Ventä, Hyrkäs, & Konttinen, 2005). The two gelatinases share similar substrate specificity of type IV collagen and other components of periodontium ECM, being regarded as having a role in extracellular degradation of denatured collagens in periodontal turnover (Ingman, Sorsa, Lindy, Koski, & Konttinen, 1994). Therefore, inhibitors of MMP-2 and MMP-9 have captured great attention in the regulation mechanisms of periodontitis.

Reversion-inducing-cysteine-rich protein with kazal motifs (RECK), a 110 kDa non-metastatic gene cloned from a v-Ki-ras-transformed NIH3T3 cell line, is a novel MMPs inhibitor that negatively regulates the synthesis and secretion of MMP-2, MMP-9 and MT1-MMP (Meng, Li, Zhang, & Sun, 2008). Previous research has demonstrated that RECK mRNA is undetectable in tumor-derived cell lines and oncogenically transformed cells while being expressed in various human tissues and untransformed cells (Takahashi et al., 1998). There was a significant inverse correlation between RECK and MMPs expression in adenocarcinoma of the lung (Takemoto et al., 2007), breast cancer (Zhang et al., 2012) as well as in oral and maxillofacial tumors such as salivary adenoid cystic carcinoma (Zhou et al., 2014). ECM degradation is the common pathological process shared by tumors and inflammations (Meng et al., 2008; Payne, Golub, Thiele, & Mikuls, 2015), similar findings have been reported in inflammatory diseases such as rheumatoid arthritis (van Lent et al., 2005) and chorioamnionitis (Benzon, Prusac, Zekic, & Vulic, 2014) apart from malignancies. Therefore, we logically proposed the hypothesis that RECK may play a role in the periodontium turnover. However, involvement of RECK/MMPs signal pathway in the ECM degration of periodontal diseases still remains to be corroborated (Liu, Zhou, & Zhu, 2016).

In the present study, we investigated the expression of RECK in human gingival tissues both healthy and diseased, and analyzed to correlate it with the production of MMP-2 and MMP-9.

2. Materials and methods

2.1. Collection of samples

All of teeth were observed and scored rigorously at six sites per tooth by a single experienced investigator. Chronic periodontitis was diagnosed according to criteria established in 1999 (Armitage, 1999). In the experimental group, the gingival tissue specimens were obtained from sites with probing depth(PD) ≥6 mm and sulcular bleeding on probing during extraction of teeth with poor periodontal prognosis. The control group biopsies were harvested from periodontally healthy sites during extractions for orthodontic purposes. Exclusion criteria included hypertension, diabetes, history of vascular diseases or other systemic diseases that might have influence on periodontium, and medicine-taken in the 6 months prior to the tooth extraction.

We used 32 chronic periodontitis samples and 20 healthy samples for immunohistochemistry staining. Gingival biopsies in this experiment were instantly fixed in 4% paraformaldehyde solution for 24 h. Meanwhile, 12 periodontally inflamed samples and 8 control samples were employed for polymerase chain reaction, for which the gingival specimens were stored at −80 °C before total RNA extraction.

2.2. Immunohistochemistry and immunohistochemical staining evaluation

All fixed gingival biopsies were embedded in paraffin, and 4 μm sections were prepared on poly-L-lysine coated slides. The slides were deparaffinized, dehydrated, immersed in 10 mM sodium citrate buffer(pH 8.0) and pretreated in microwave, followed by 10-min rinse with phosphate-buffered saline(PBS). The slides were blocked with 3% hydrogen peroxide for 25 min at room temperature, and then 5% BSA(Roche, Switzerland) for 20 min. Afterwards, the slides were incubated at 4 °C overnight with primary antibodies: anti-RECK(1:50 goat MAb, Santa Cruz Biotechnology, USA), anti-MMP-2 and anti-MMP-9(1:200 mouse MAb, R&D, USA). Then the slides were stained with the 2-step plus Poly-HRP Anti-Goat IgG Detection System (Dako, Denmark) for RECK or 2-step plus Poly-HRP Anti-Mouse IgG Detection System (R&D, USA) for MMP-2 and MMP-9. After visualization of the reaction with DAB, the sections were counterstained with Mayer's hematoxylin, dehydrated with increasing concentration of ethanol series, cleared with xylene and mounted with glycerol. Primary antibody was substituted with PBS for negative controls to confirm its specificity. Besides, one dewaxed slide of each biopsy was stained with hematoxylin and eosin before immunostaining, then the H&E stained sections were dehydrated, cleared and coverslipped.

Sections were evaluated by an experienced investigator who was blinded to the experimental group. The positive signals of expression of RECK, MMP-2 and MMP-9 protein were classified by pale yellow, buff or brown particles located in intracytoplasm. Evaluation of immunohistochemical staining was in compliance with intensity and proportion. The intensities were assessed as 0 for no staining, 1 for mild staining, 2 for moderate staining and 3 for intense staining; while the proportion of cells stained was scored in a semiquantitative scale: 0, <5%; 1, 6–25%; 2, 26–50%; and 3, >50%. Finally the multiplied score(intensity plus extension) was classified as follows: <2, negative(−); 2–3, positive(+); and ≥4, strong positive(++). A combined score equaling or exceeding (+) was defined as positive expression (Zhang, Zhang, Xu, & Xie, 2009).

2.3. Polymerase chain reactions(PCR) and image analysis

Total RNA was isolated from 100 mg sample tissues by Trizol reagent(Invitrogen Life Technologies, USA) according to the instructions. PCRs were carried out in 96-well plates in a total volume of 25 μl, including 2.5 μl of cDNA and 2.0 μl of primers (7.5 μl) (Table 1). Quantitation was assessed by densitometry, with GAPDH used as internal control for PCR reactions and the products analyzed on a 3.0% agarose minigel system(Aragose G-10, Biowest, Spain). All reactions were carried out in duplicate.

A computerized image analysis system(Tanon 1600-R, Shanghai, China) was utilized to quantify the band intensity. Results were evaluated as a relative unit determined by normalization of the optical density(OD) of RECK or MMP-2, −9 band to that of GAPDH band respectively(e.g. the ratio of RECK/GAPDH).

2.4. Statistical analyses

Results of RECK, MMP-2 and MMP-9 protein expression are described with positive percentage(PP), with differences between groups tested for significance using the Kruskal-Wallis H test and correlations tested through Spearman's rank test.

RECK, MMP-2 and MMP-9 mRNA expression are expressed as mean ± standard deviation(SD), and *T*-test was employed to

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