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ORIGINAL ARTICLE

Intracellular localization of *Treponema denticola* chymotrypsin-like proteinase in chronic periodontitis

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Treponema denticola is an important periodontal pathogen capable of tissue invasion. Its chymotrypsin-like proteinase (CTLP) can degrade a number of basement membrane components *in vitro*, thus suggesting a contribution to tissue invasion by the spirochete. The aim of this study was to analyze the localization of CTLP in chronic periodontitis tissues *ex vivo*. A polyclonal antibody specific to *T. denticola* cell-bound CTLP was used to detect the spirochetes in the gingival tissues of patients with moderate to severe chronic periodontitis (n = 25) by immunohistochemistry and periodic acid-Schiff staining (PAS). The presence of *T. denticola* in the periodontal tissue samples was analyzed by PCR. Periodontal tissue samples of 12 of the 25 patients were found to be positive for *T. denticola* by PCR. Moreover, CTLP could be detected in the periodontal tissues of all these patients by immunohistochemistry. In the epithelium, the CTLP was mostly intracellular. Typically, the positive staining could be seen throughout the whole depth of the epithelium. When detected extracellularly, CTLP was localized mainly as granular deposits. The connective tissue stained diffusely positive in four cases. The positive staining co-localized with the PAS stain in nine cases. *T. denticola* and its CTLP could be detected in diseased human periodontium both intra- and extracellularly. The granular staining pattern was suggestive of the presence of *T. denticola* bacteria, whereas the more diffused staining pattern was indicative of the recent presence of the bacterium and shedding of the cell-bound proteinase.

Keywords: *dentilisin*; *spirochetes*; *major outer sheath protein*

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The microbial etiology of chronic periodontitis is complex and specific bacterial species have been associated with the disease (1). Anaerobic spirochetes are known human pathogens and often represent the predominant organisms in severe forms of periodontal disease. Although they may be found in small amounts in the normal oral flora, their proportion increases significantly in the gingival pockets during periodontal infection (2). *Treponema denticola*, the most frequently isolated and studied oral spirochete, is often found in large numbers at diseased periodontal sites, and it is particularly associated with severe and refractory periodontal disease (3–5). It possesses a variety of pathogenic properties, such as adhesion to epithelial cells and extracellular matrix components, production of tissue-destructive enzymes, secretion of cytotoxic products, suppression of local immune responses, and invasion of gingival epithelial cells (2, 6, 7). It has been shown to remain viable within the host cell for several hours by resisting endolysosomal degradation (7). Furthermore, *T. denticola* has the ability to adhere to other bacteria and it is most often isolated with other oral microorganisms, especially *Porphyromonas gingivalis* (8, 9). Adhesion of *T. denticola* to *P. gingivalis* and *Fusobacterium nucleatum*, as well as to a range of host

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protein molecules, is mediated by a major outer sheath (Msp) glycoprotein of the organism (10).

T. denticola chymotrypsin-like proteinase (CTLP), also called dentilisin, is a cell-bound enzyme, which is released from the *T. denticola* outer cell membrane (6). It has been shown to be able to degrade host proteins and has been suggested to contribute to tissue invasion by the spirochete (11, 12). It has also been found to mediate the adherence of *T. denticola* to other periodontal pathogens such as *P. gingivalis* (13). The proteinase can rapidly penetrate through cell layers and cause increased permeability of the epithelium. The CTLP degrades a number of structural proteins, including gelatin, laminin, and fibronectin (14, 15). Even though this enzyme has attracted the interest from several groups, little is known of its expression and tissue distribution.

The primary aim of this study was to analyze the localization of the *T. denticola* CTLP in chronic periodontitis tissues *ex vivo*. Our secondary aim was to study the association of the CTLP with the presence of *P. gingivalis* and *Candida albicans*. Our hypothesis was that *T. denticola* CTLP could be detected in *T. denticola*–infected tissue samples by immunohistochemistry and that as an important virulence factor its expression correlated with the detection presence of *T. denticola* and *P. gingivalis* by PCR.

Materials and methods

Patients and samples

Surgical samples of diseased gingiva were obtained from patients with moderate to severe generalized adult type chronic periodontitis (CP; n = 25; 15 women and 10 men; 53.4 ± 11.0 years) as judged by clinical measurements of pocket depths, loss of attachment, radiographic bone loss, suppuration, and gingival bleeding on probing. The patients had radiographic evidence of bone loss of 20–50% on many teeth and a mean loss of attachment ranging from 4 to 6 mm. Samples were obtained from premolar–molar regions during flap surgery of the initial periodontal therapy. All specimens were formalin-fixed and paraffin-embedded. All the 25 samples had previously been tested for *Candida albicans* infection (16). In addition, 13 of these samples had previously also been tested for *P. gingivalis* infection (17). The study was approved by the Ethical Committee of the Institutes of Dentistry, University of Helsinki, and the subjects were enrolled into the study and treated in compliance with the Helsinki Agreement as revised in 1983.

Buffers and reagents

Polymerase chain reaction (PCR) primers (5′TAA TAC CGA ATG TGC TCA TTT ACA T and 5′TCA AAG AAG CAT TCC CTC TTC TTC TTA) specific for *T. denticola* 16S rRNA were used for the detection of the bacterium. DNA polymerase (Dynazyme II, Finnzymes, Espoo, Finland) was applied for amplification with the reaction buffer (50 mM KCl, 10 mM tris-HCl; pH 8.8; 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 μM of each dNTP). For the immunohistochemical staining, a pure in-house rabbit polyclonal antibody (IgG; designated CHR) against *T. denticola* chymotrypsin-like protein was used as a primary antibody as described earlier (11). Briefly, the CTLP was isolated from a sonicated cell extract of *T. denticola* ATTC 35405 by preparative sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The purified CTLP was injected intramuscularly into a New Zealand White rabbit with complete Freund adjuvant. Subsequent intramuscular injections, without adjuvant, were done on days 8, 14, 22, 36, and 50. The rabbit was bled via the marginal ear vein on day 57. The purified immunoglobulin G (IgG) fraction was prepared by passing the antiserum through a column of protein A-Sepharose CL 4B (Sigma Chemical Co., St. Louis, MO). The sample was exhaustively washed on the column with 0.1 M borate-0.5 M NaCl buffer (pH 8.4). IgG was then eluted with 0.1 M glycine-0.5 M NaCl buffer (pH 2.5), followed by dialysis against 50 mM phosphate-buffered saline (PBS; pH 7.2).

PCR for detection of T. denticola

The presence of *T. denticola* in the periodontal tissue samples was analyzed by PCR. Five 10-μm-thick, paraffin-embedded sections of each sample were homogenized mechanically. A hot start PCR method was used with specific *T. denticola* primers according to Ashimoto and coworkers (18). *T. denticola* ATCC 35405 was used as a positive control. DNA polymerase was applied for amplification with the reaction buffer. The PCR amplification was performed in a DNA thermal cycler (Cycler 480, PerkinElmer Corporation, Waltham, MA). Before the PCR cycles the reaction components without the enzyme were kept at 96°C for 1 min and cooled to 80°C, at which temperature DNA polymerase (DynaZyme, FinnZymes, Espoo, Finland) was added to each tube. The PCR products were visualized by UV light after electrophoresis on agarose gel containing ethidium bromide.

Immunohistochemical staining

Formalin-fixed, 4-μm-thick paraffin-embedded sections were deparaffinized in xylene and rehydrated in graded alcohol series and water. Sections were incubated in pepsin and washed in PBS. Endogenous peroxidase activity was inhibited with 0.3% H_2O_2 in methanol; then the sections were washed with PBS. A modification of the Vectastain® Kit (Vector Laboratories, Burlingame, CA) protocol was used as described earlier (16, 19). Rabbit polyclonal antibody (designated CHR) against *T. denticola* CTLP was used as a primary antibody (6, 11). Control stainings were performed by omitting the primary antibody and Anti-*Helicobacter pylori* rabbit monoclonal

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antibody (Ventana, Tucson, AZ) was used as a control primary antibody. Finally, the slides were mounted with Glysergel (DAKO Glostrup, Denmark) The specimens were examined with Olympus BX light microscope (Olympus Optical, Tokyo, Japan) and photographed with an Olympus digital camera.

Periodic acid-Schiff stain

The presence of *T. denticola* Msp was analyzed with PAS stain (10). Formalin-fixed, 4-μm-thick paraffin-embedded sections were deparaffinized in xylene and rehydrated in graded alcohol series and in water. Before staining with Schiff's leucofuchsin reagents, the sections were first exposed to periodic acid.

Data analyses

Data were analyzed by using GraphPad Prism version 5.00 (GraphPad Inc., San Diego, CA). The two-tailed Mann Whitney test and Fisher's exact test were used for the comparisons between groups. P-values of less than 0.05 were considered statistically significant.

Results

Histopathological samples of diseased gingiva were obtained from 25 patients with moderate to severe CP. Twelve of the 25 periodontal tissue samples (48%) were found to be positive for *T. denticola* by PCR. *T. denticola* CTLP could be detected in the periodontal tissues of all the PCR positive samples by immunohistochemistry. Positive staining of CTLP was detected in the gingival tissues of three additional patients repeatedly negative of *T. denticola* by PCR. The areas of positive staining were very limited in these patients.

The positive immunohistochemical staining for CTLP localized into the epithelium in 10 of the 12 samples (83%) positive for *T. denticola* by PCR. Of these, seven showed only staining of the epithelium (pocket n = 1, oral n = 4, or both n = 1) and three showed staining in both epithelium and the connective tissue. In the samples that were *T. denticola* positive by PCR, the positive CTLP staining localized into the epithelium in 83% of the samples (10 of 12). Two of these showed staining of both the epithelium and the underlying connective tissue and eight only of the epithelium. In two of the samples, the positive staining was detected only in the connective tissue. Staining of dental plaque could be seen in 90% of the PCR positive samples (Fig. 1e). The two PCR negative samples that had positive staining of the connective tissue also showed positive staining of plaque.

In the epithelium, *T. denticola* CTLP was expressed in a patchy manner and could mainly be seen intracellularly (Fig. 1). Typically, the positive staining could be detected in bands extending throughout the whole depth of the epithelium. In these areas, the tissue structure appeared looser and irregular (Fig. 2). The positive staining co-

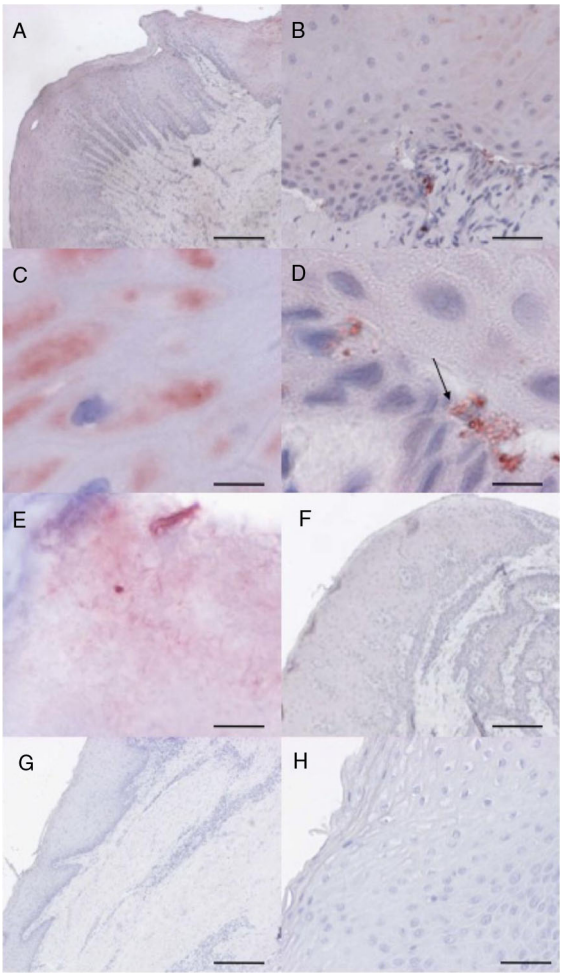

Fig. 1. Localization of *Treponema denticola* CTLP in human periodontal tissues. (A) *T. denticola* chymotrypsin-like protein was expressed throughout the oral epithelium in a patchy manner as detected by the rabbit polyclonal antibody (A–C). The *T. denticola* CTLP could mainly be detected in the epithelium and in the dental plaque (E) in most tissue samples. The underlying connective tissue was mostly negative for the protein. The *T. denticola* CTLP was expressed intracellularly (B, C) and could be seen extracellularly mainly in granular deposits suggestive of presence of *T. denticola* bacteria (D, arrow). Controls: control stainings were performed on *T. denticola* PCR negative chronic periodontitis tissue (F), on *T. denticola*-infected oral epithelium by omitting the primary antibody (G), and on *T. denticola*-infected oral epithelium probed or by replacing the primary antibody with an irrelevant anti-*Helicobacter pylori* rabbit monoclonal antibody (Ventana, Tucson, AZ) (H). Samples (A)–(D) are from a PCR-positive patient Panels (A)–(D) were probed with anti-CTLP antibody.
Original magnifications: (A, F, G), 4×; (B), 20×; (C–E), 40×.
Scale bars: (A, F, G), 200 μm, (B, H), 50 μm, (C, D, E), 10 μm.

localized with the PAS stain suggesting the presence of *T. denticola* Msp protein. When detected extracellularly *T. denticola* CTLP was localized mainly as granular deposits (Fig. 1).

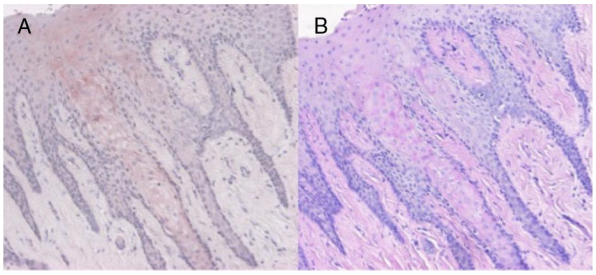

Fig. 2. A wake of *Treponema denticola* in the epithelium. (A) A wake of *T. denticola* chymotrypsin-like protein can be seen throughout the oral epithelium as detected by the rabbit polyclonal antibody. The tissue structure in the area of positive staining appears loose and irregular. (B) A similar area of altered tissue structure can be seen by PAS stain. The PAS staining suggested the presence of the *T. denticola* Msp protein, which was supported by the positive staining obtained with the antibody. However, this can also be due to PAS stain binding to adjacent host glycosylated proteins.

The tissue samples used in this study had previously been analyzed for the presence of *C. albicans* in periodontal tissue (16). Four of the samples were positive for *C. albicans*. These four samples were all negative for *T. denticola* by PCR but two showed repeatedly positive staining for *T. denticola* CTLP in dental plaque and tissue.

Thirteen of the tissue samples in this study had been previously studied previously for the presence of *P. gingivalis* by PCR and immunohistochemistry (17). Of these 13 samples, 10 were positive for *P. gingivalis* (77%). Of the 10 tissue samples positive for *P. gingivalis* three were positive for *T. denticola* by PCR (30%) and six for CTLP (60%) in the present study. Two samples were positive for *T. denticola* but negative for *P. gingivalis* and one sample was negative for both *T. denticola* and *P. gingivalis*.

The differences were not statistically significant ($P = 0.2$).

Discussion

In this study, we demonstrated the intracellular localization of *T. denticola* CTLP in gingival epithelium. The proteinase could be detected in all the periodontal tissue samples that were PCR positive for *T. denticola*. Positive staining of CTLP could be seen throughout the whole depth of the epithelium and was expressed in a patchy manner. The positive staining co-localized with the PAS stain suggested the presence of *T. denticola* Msp protein. However, this can also be due to PAS stain binding to adjacent host glycosylated proteins. The intracellular localization of *T. denticola* has been shown in previous studies and it has been found to resist endolysosomal degradation within the host cell for as long as 48 h (7). According to our results CTLP, a highly active proteinase, is likewise found intracellularly in considerable amounts. Human saliva has been shown to inhibit CTLP (20). However *T. denticola* can mostly be seen in subgingival plaque and in the depth of the periodontal pocket where it is out of reach from the saliva (8, 21).

In the areas with CTLP staining a loss of tissue integrity could be seen, as the tissue structure appeared looser and irregular. There is evidence that CTLP can contribute to tissue destruction (14, 22–24). It disrupts epithelial layers by breaking epithelial tight junctions (12). Although the strongest staining of CTLP was mainly seen in the dental plaque and epithelial layers, CTLP was detected throughout the whole tissue – from dental plaque all the way to the connective tissue. The high amount of CTLP in plaque could help in the degradation of tissue in the gingival pocket and cause increased permeability of the epithelium and provide a route for infection and invasion of *T. denticola* and other periodontal pathogens. *P. gingivalis* and *T. denticola* have demonstrated synergy in the formation of polymicrobial biofilms (25). In addition, nutritional interactions between these two species have been reported (26). CTLP has been shown to mediate the adherence of *T. denticola* with *P. gingivalis* (13). Thirteen of the tissue samples in this study had been previously studied for the presence of *P. gingivalis* by PCR and immunohistochemistry (17). Of the 10 tissue samples positive for *P. gingivalis*, six were positive for CTLP (60%) in the present study. Two samples were positive for *T. denticola* but negative for *P. gingivalis* and one sample was negative for both *T. denticola* and *P. gingivalis*. The differences were statistically not significant.

In our study 60% of the samples positive for *P. gingivalis* were also positive for CTLP but only 30% for *T. denticola*.

CTLP modulates the function of polymorphonuclear leukocytes through the activation of complement (27). This in turn leads to the release of elastase, cathepsin G and matrix metalloproteinases (MMP)-8 and -9, which have been found in increased amounts in *T. denticola* positive infection, and are clearly involved in tissue destruction during periodontal disease (28). *T. denticola* chymotrypsin-like protease can directly activate human proMMP-1 and -8 (29). Doxycycline has been shown to inhibit *T. denticola* trypsin but not CTLP (30). However, chlorhexidine inhibits the catalytic activity of MMP-8 and -9 (31).

It has been previously demonstrated that *T. denticola* remains intracellularly in endosome-like structures and not in the cytosol (7). When detected extracellularly, CTLP was localized mainly as granular deposits. The major intracellular actions of CTLP are not yet known and this is of future interest. Unlike viral particles, *T. denticola* CTLP was not detected in the nucleus, therefore it should not directly impact on the viability of the cells. However, it could be presumed that it affects the protein synthesis of the cell. Our findings are in line with

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these results suggesting that CTLP is expressed by *T. denticola* during tissue and cell invasion. Interestingly, CTLP staining was often diffuse and not only limited to structures suggestive of the presence of *T. denticola*.

This staining pattern is more suggestive of shedding of the protein during tissue invasion, which can contribute to its virulence.

Conclusion

In conclusion, our study demonstrated that *T. denticola* CTLP is mainly found intracellularly in the periodontal tissue. The proteinase was seen in dental plaque and in all tissue compartments. CTLP staining was often diffuse and not only limited to structures suggestive of the presence of *T. denticola*. This indicates that CTLP has an active role in chronic periodontal disease by degrading tissue in several layers. Previous studies have demonstrated intracellular localization of *T. denticola*. Our study highlights the active role of this pathogen inside the cell through high production of this proteolytic enzyme.

Conflict of interest and funding

This research was funded by a grant from the Paulo Foundation and the Finnish Medical Society Duodecim. The authors report no conflicts of interest related to this study.

References

1. Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner AC, Yu WH, et al. The human oral microbiome. J Bacteriol 2010; 192: 5002–17.
2. Chan EC, McLaughlin R. Taxonomy and virulence of oral spirochetes. Oral Microbiol Immunol 2000; 15: 1–9.
3. Sela MN. Role of *Treponema denticola* in periodontal diseases. Crit Rev Oral Biol Med 2001; 12: 399–413.
4. Haffajee AD, Yaskell T, Torresyap G, Teles R, Socransky SS. Comparison between polymerase chain reaction-based and checkerboard DNA hybridization techniques for microbial assessment of subgingival plaque samples. J Clin Periodontol 2009; 36: 642–9.
5. You M, Mo S, Leung WK, Watt RM. Comparative analysis of oral treponemes associated with periodontal health and disease. BMC Infect Dis 2013; 13: 174.
6. Uitto VJ, Grenier D, Chan EC, McBride BC. Isolation of a chymotrypsin-like enzyme from *Treponema denticola*. Infect Immun 1988; 56: 2717–22.
7. Shin J, Choi Y. The fate of *Treponema denticola* within human gingival epithelial cells. Mol Oral Microbiol 2012; 7: 471–82.
8. Kigure T, Saito A, Seida K, Yamada S, Ishihara K, Okuda K. Distribution of *Porphyromonas gingivalis* and *Treponema denticola* in human subgingival plaque at different periodontal pocket depths examined by immunohistochemical methods. J Periodontal Res 1995; 30: 332–41.
9. Scapoli L, Girardi A, Palmieri A, Testori T, Zuffetti F, Monguzzi R, et al. Microflora and periodontal disease. Dent Res J 2012; 9: S202–6.
10. Rosen G, Genzler T, Sela MN. Coaggregation of *Treponema denticola* with *Porphyromonas gingivalis* and *Fusobacterium nucleatum* is mediated by the major outer sheath protein of *Treponema denticola*. FEMS Microbiol Lett 2008; 289: 59–66.
11. Grenier D, Uitto V-J, McBride B. Cellular location of a *Treponema denticola* chymotrypsin-like protease and importance of the protease in migration through the basement membrane. Infect Immun 1990; 58: 347–51.
12. Chi B, Qi M, Kuramitsu HK. Role of dentilisin in *Treponema denticola* epithelial cell layer penetration. Res Microbiol 2003; 154: 637–43.
13. Cogoni V, Morgan-Smith A, Fenno JC, Jenkinson HF, Dymock D. *Treponema denticola* chymotrypsin-like proteinase (CTLP) integrates spirochaetes within oral microbial communities. Microbiology 2012; 158: 759–70.
14. Bamford CV, Fenno JC, Jenkinson HF, Dymock D. The chymotrypsin-like protease complex of *Treponema denticola* ATCC 35405 mediates fibrinogen adherence and degradation. Infect Immun 2007; 75: 4364–72.
15. Miao D, Fenno JC, Timm JC, Joo NE, Kapila YL. The *Treponema denticola* chymotrypsin-like protease dentilisin induces matrix metalloproteinase-2 dependent fibronectin fragmentation in periodontal ligament cells. Infect Immun 2011; 79: 806–11.
16. Järvensivu A, Hietanen J, Rautemaa R, Sorsa T, Richardson M. *Candida* yeasts in chronic periodontitis tissues and subgingival microbial biofilms in vivo. Oral Dis 2004; 10: 106–12.
17. Rautemaa R, Järvensivu A, Kari K, Wahlgren J, DeCarlo A, Richardson M, et al. Intracellular localization of *Porphyromonas gingivalis* thiol proteinase in periodontal tissues of chronic periodontitis patients. Oral Dis 2004; 10: 298–305.
18. Ashimoto A, Chen C, Bakker I, Slots J. Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced periodontitis lesions. Oral Microbiol Immunol 1996; 11: 266–71.
19. Järvensivu A, Rautemaa R, Sorsa T, Richardson M. Specificity of the monoclonal antibody 3H8 in the immunohistochemical identification of *Candida* species. Oral Dis 2006; 12: 428–33.
20. Rosen G, Sela MN, Bachrach G. The antibacterial activity of LL-37 against *Treponema denticola* is dentilisin protease independent and facilitated by the major outer sheath protein virulence factor. Infect Immun 2012; 8: 1107–14.
21. Li J, Helmerhorst EJ, Leone CW, Troxler RF, Yaskell T, Haffajee AD, et al. Identification of early microbial colonizers in human dental biofilm. J Appl Microbiol 2004; 97: 1311–8.
22. Grenier D. Degradation of host protease inhibitors and activation of plasminogen by proteolytic enzymes from *Porphyromonas gingivalis* and *Treponema denticola*. Microbiology 1996; 142: 955–61.
23. Ding Y, Uitto VJ, Haapasalo M, Lounatmaa K, Konttinen YT, Salo T, et al. Membrane components of *Treponema denticola* trigger proteinase release from human polymorphonuclear leukocytes. J Dent Res 1996; 75: 1986–93.
24. Lux R, Miller JN, Park NH, Shi W. Motility and chemotaxis in tissue penetration of oral epithelial cells layer by *Treponema denticola*. Infect Immun 2001; 69: 6276–83.
25. Zhu Y, Dashper SG, Chen YY, Crawford S, Slakeski N, Reynolds EC. *Porphyromonas gingivalis* and *Treponema denticola* synergistic polymicrobial biofilm development. PLoS One 2013; 8: e71727.
26. Grenier D. Nutritional interactions between two suspected periodontopathogens, *Treponema denticola* and *Porphyromonas gingivalis*. Infect Immun 1992; 60: 5298–301.
27. Yamazaki T, Miyamoto M, Yamada S, Okuda K, Ishihara K. Surface protease of *Treponema denticola* hydrolyzes C3 and influences function of polymorphonuclear leukocytes. Microbes Infect 2006; 8: 1758–63.

28. Yakob M, Meurman JH, Sorsa T, Söder B. *Treponema denticola* associates with increased levels of MMP-8 and MMP-9 in gingival crevicular fluid. Oral Dis 2013; 19: 694–701.
29. Sorsa T, Ingman T, Suomalainen K, Haapasalo M, Konttinen YT, Lindy O, et al. Identification of proteases from periodontopathogenic bacteria as activators of latent human neutrophil and fibroblast-type interstitial collagenases. Infect Immun 1992; 60: 4491–95.
30. Grenier D, Plamondon P, Sorsa T, Lee HM, McNamara T, Ramamurthy NS, et al. Inhibition of proteolytic, serpinolytic, and progelatinase-b activation activities of periodontopathogens by doxycycline and the non-antimicrobial chemically modified tetracycline derivatives. J Periodontol 2002; 73: 79–85.
31. Gendron R, Grenier D, Sorsa T, Mayrand D. Inhibition of the activities of matrix metalloproteinase-2, -8 and -9 by chlorhexidine. Clin Diag Lab Immunol 1999; 6: 437–9.

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