

Stimulation of Human Periodontal Ligament Fibroblasts Using Purified Dentilisin Extracted from *Treponema denticola*

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Abstract

Periodontal disease is a chronic multifactorial disease triggered by a complex of bacterial species. These interact with host tissues to cause the release of a broad array of pro-inflammatory cytokines, chemokines, and tissue remodelers, such as matrix metalloproteinases (MMPs), which lead to the destruction of periodontal tissues. Patients with severe forms of periodontitis are left with a persistent pro-inflammatory transcriptional profile throughout the periodontium, even after clinical intervention, leading to the destruction of teeth-supporting tissues. The oral spirochete, *Treponema denticola*, is consistently found at significantly elevated levels at sites with advanced periodontal disease. Of all *T. denticola* virulence factors that have been described, its chymotrypsin-like protease complex, also called dentilisin, has demonstrated a multitude of cytopathic effects consistent with periodontal disease pathogenesis, including alterations in cellular adhesion activity, degradation of various endogenous extracellular matrix-substrates, degradation of host chemokines and cytokines, and ectopic activation of host MMPs. Thus, the following model of *T. denticola*–human periodontal ligament cell interactions may provide new knowledge about the mechanisms that drive the chronicity of periodontal disease at the protein, transcriptional, and epigenetic levels, which could afford new putative therapeutic targets.

Keywords: Periodontal disease, Host–microbe interactions, Periodontal ligament cells, *Treponema denticola*, Dentilisin

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Background

The human periodontal ligament serves as a connector between the tooth and the surrounding alveolar bone proper. Its main role is to convert mechanical forces into chemical signals, which largely mediate tissue turnover through the expression of various proteases, primarily matrix metalloproteinases (MMPs) (Ho et al., 2007; Sokos et al., 2015; Takimoto et al., 2015; Jiang et al., 2016; Lin et al., 2017; Kim et al., 2020). Periodontal diseases are caused by bacterially derived factors, leading to a chronic infectious inflammatory disease that affects the periodontium and gradually destroys the tooth-supporting alveolar bone (Sela et al., 1997; Asai et al., 2003; Hayashi et al., 2010; Trindade et al., 2014; Cecil et al., 2017; Deng et al., 2017; Gao et al., 2020) (Figure 1). As periodontal disease progresses, periodontopathogenic bacteria invade deeper into the subgingival space, compromising the periodontal ligament (PDL) function and contributing to tooth loss.

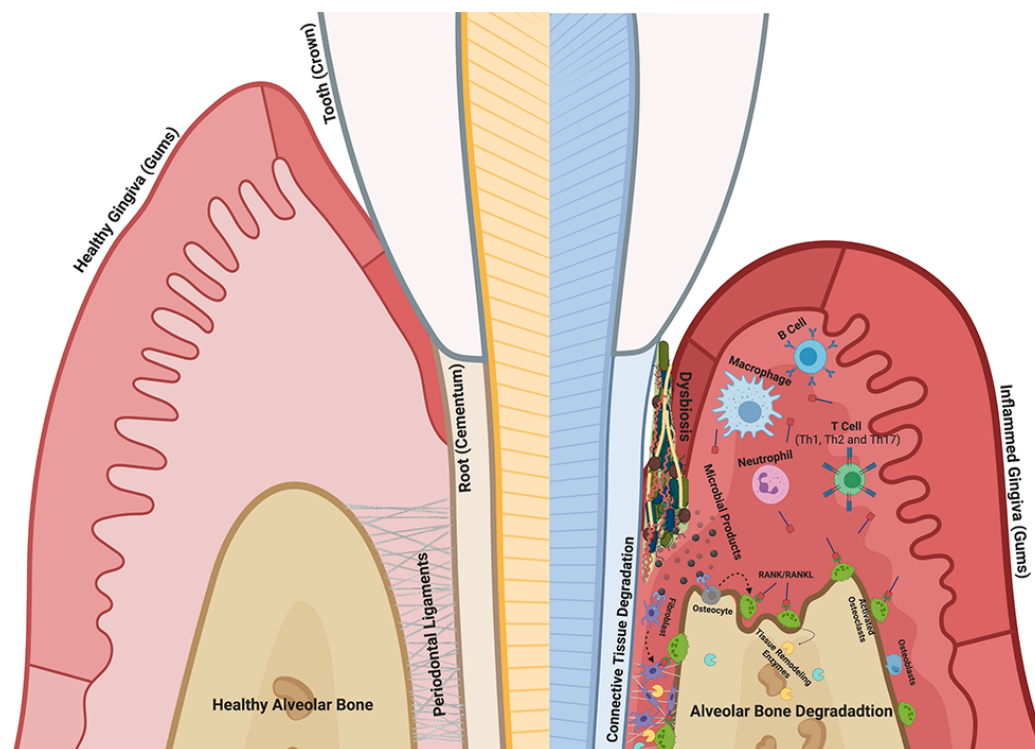


Figure 1. Pathophysiology of periodontal disease

Among over 500 species of bacterium, the oral spirochete, *Treponema denticola*, is consistently found at significantly elevated levels in advanced lesions (Ateia et al., 2018; Solbiati and Frias-Lopez, 2018). Additionally, elevated *T. denticola* biofilm levels, combined with elevated MMP levels in host tissue, display robust combinatorial characteristics in predicting advanced periodontal disease severity. Thus, clinical data regarding the increased presence of *T. denticola* in periodontal lesions, together with basic research results involving the role of *T. denticola* products, suggest that it plays a pivotal role in driving periodontal disease progression. Therefore, delineation of causative mechanisms that *T. denticola* uses to drive host modulation may help to identify novel targets for better or alternative treatments for this chronic disease.

Conserved lipid moieties of the protease complex recognized by host receptor complexes may contribute to the activation of innate immune responses (Schenk et al., 2009). Because predominant host responses to lipoproteins are believed to be to their lipid moieties, most studies have focused on diacylated lipopeptide, Pam2CSK4, and triacylated lipopeptide, Pam3CSK4, which mimic bacterial lipoproteins for their potent immunostimulatory and osteoclastogenic activities (Schenk et al., 2009; Kim et al., 2013; Wilson and Bernstein, 2016). Recent studies have demonstrated that synthetic di- and tri-acylated lipopeptides, which preferentially activate TLR2/6 and TLR2/1-

dependent pathways respectively, are sufficient to induce alveolar bone loss in mice (Kim et al., 2013; Souza et al., 2020), broadening the avenues of investigation into the role of lipoproteins underpinning the pathogenesis of periodontal disease. However, studies that utilize endogenously expressed bacterial lipopeptides are lacking.

Several proteinases and peptidases secreted by *T. denticola* have been identified as causative factors that likely contribute to periodontal disease pathogenesis, due to their roles in processing host tissue proteins and peptides to fulfill the nutritional requirements of these highly motile and invasive organisms (Veith et al., 2009; Ellis and Kuehn, 2010; Visser et al., 2011; Asai et al., 2003; Cecil et al., 2016). Of all *T. denticola* surface-expressed proteins that have been described, a chymotrypsin-like protease complex, also called dentilisin, has demonstrated a multitude of cytopathic effects consistent with periodontal disease pathogenesis, including changes in cellular adhesion activity (Bamford et al., 2007; Sano et al., 2014), degradation of various endogenous extracellular matrix-substrates (Bamford et al., 2007; Miao et al., 2011; Inagaki et al., 2016), and degradation of host chemokines and cytokines (Miyamoto et al., 2006; McDowell et al., 2012). Despite the many studies demonstrating its role at the protein level, few direct links have been reported between the activity of *T. denticola*'s protease and the cellular and tissue processes driving periodontal tissue destruction. The following protocol delineates the experimental setup to study the interactions between *T. denticola*'s dentilisin protease and human periodontal ligament cells.

Materials and Reagents

1. 6-well clear multi-well plate (Fisher Scientific, catalog number: 25373-187)
2. 10 cm Falcon tissue culture plate (The Lab Depot, catalog number: 25373-10)
3. Minimal essential medium- α (MEM- α) (ThermoFisher Scientific, catalog number: 12571063)
4. Phosphate buffered saline (PBS) (Thermo Fisher Scientific, catalog number: 14190-094)
5. Penicillin/streptomycin (P/S) (Thermo Fisher Scientific, Gibco, catalog number: 15140122)
6. Amphotericin B (Thermo Fisher Scientific, Gibco, catalog number: 15290018)
7. 0.25% trypsin with phenol red (Thermo Fisher, catalog number: 25200-05)
8. Heat-inactivated fetal bovine serum (FBS) (Gibco, catalog number: 10-438-026)
9. BCA protein assay kit (Millipore Sigma, catalog number: 71285-3)

Equipment

1. 96-well SpectraMax Plus microplate reader (VWR, catalog number: 89212-396)
2. Labomed Lx400 phase contrast HD digital microscope (Microscope Central, catalog number: 9126017T-HDS)
3. SteriCycle 370 CO₂ incubator (Thermo Fisher Scientific, catalog number: TH-370N)
4. NanoDrop One Microvolume UV-Vis spectrophotometer (Fisher Scientific, catalog number: 13-400-519)
5. Bright line hemacytometer chamber (Carolina, catalog number: 700722)
6. Thermo Sorvall ST16R refrigerated centrifuge (Thermo Fisher Scientific, catalog number: 75-004-240)

Procedure

Human periodontal ligament cell cultures (hPDL)

1. As described previously (Scanlon et al., 2011), prepare the primary culture of human periodontal ligament cells via the direct cell outgrowth method, by isolating cells from the periodontal ligament tissue around the middle third of healthy human teeth extracted. Maintain cells in approximately 10 mL of MEM- α augmented with 10% FBS, 1% P/S, and 1% amphotericin B in 10 cm Falcon tissue culture plates in a humid atmosphere with 95% air and 5% CO₂ at 37 °C.
 - a. Passage cell outgrowths when they reach approximately 90% confluency using 0.25% trypsin and a Thermo Sorvall ST16R refrigerated centrifuge. Although variation will be observed from patient to patient,

cells usually take approximately three to four days to become confluent after seeding cells at approximately 70%.

- b. Use cells passaged three to six times for experimentation.
- c. Validate human periodontal ligament cell parameters by 1) measuring mRNA expression of a specific isoform of the POSTN gene, which is exclusively expressed by periodontal ligament cells, 2) evaluating cell morphology, and 3) examining expression of other confident cell biomarkers, such as vimentin (general fibroblast marker) and *CD45*, which ensure cultures are free of macrophages (Marchesan et al., 2011).

If cultures are contaminated with other cell types, hPDL cells can be further purified via FACS sorting using the biomarkers reported above (Basu et al., 2010).

2. Seed approximately 9×10^5 cells into 6-well clear multi-well plates and allow them to adhere in the presence of MEM- α with 10% FBS, 1% amphotericin B, and 1% P/S for approximately 24 h before stimulating or challenging the cells.

Stimulation of human periodontal ligament cells using purified dentilisin

Use a BCA protein assay kit according to the manufacturer's recommendations to determine purified dentilisin (UniProt Accession #: P96091) sample concentrations

3. Scan the plates using a 96-well SpectraMax Plus Microplate Reader.
4. Determine enzymatic activity using gelatin zymography as described previously (Cathcart, 2016).
5. Wash cells with PBS 2–3 times.
6. Add the purified dentilisin/PBS solution to MEM- α media with no phenol red, no serum, and no antibiotics to a final concentration of 1 $\mu\text{g/mL}$.
7. Add this dentilisin solution to healthy human periodontal ligament cell cultures and incubate in a humid atmosphere containing 95% air and 5% CO_2 at 37 °C for 2 h.
Stimulation with purified dentilisin will cause significant changes to the cells' morphology (Figure 2).
8. Gently wash cells with PBS twice and incubate in a humid atmosphere containing 95% air and 5% CO_2 at 37 °C for an additional 22 h in MEM- α with no FBS, P/S, or amphotericin B.

Data analysis

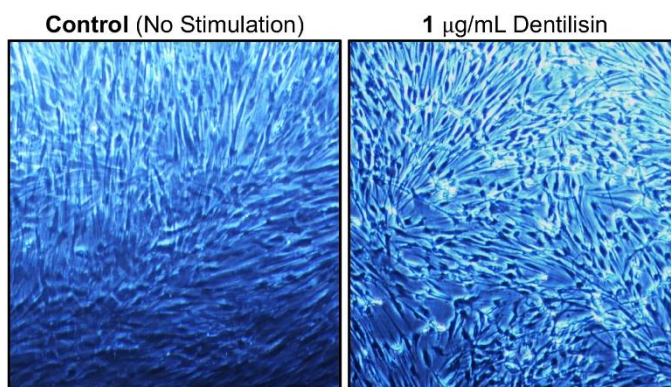


Figure 2. Morphological Change of hPDL Cells Following Purified Dentilisin Stimulation. Healthy human periodontal ligament fibroblasts were challenged with purified dentilisin at a final concentration of 1 $\mu\text{g/mL}$ for 2 h in MEM- α without serum or antibiotics, followed by a 22 h incubation in a humid atmosphere containing 95% air and 5% CO_2 at 37 °C in MEM- α media with no supplementation.

Following this experimental setup, harvest cells for analysis of various parameters. In our studies, we harvested cells or conditioned media to evaluate MMP enzymatic activity (gelatin zymography) and protein and RNA expression (Western Blot and qRT-PCR), and for cell imaging (immunofluorescence) (Ganther et al., 2021).

Notes

If collecting conditioned media samples, it is highly recommended to use media without phenol red, serum, or antibiotics as dentilisin's protease activity can cleave numerous substrates and may interfere with protein concentration measurements.

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Competing interests

The authors declare no conflict of interest.

Ethics

Approval to conduct human subjects' research was obtained from the University of California San Francisco Institutional Review Board (# 16-20204; reference #227030).

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