Effect of *Treponema Denticola* Infection on Epithelial Cells

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Abstract

Chronic periodontitis is an infectious disease caused by periodontopathic bacteria in subgingival plaque. One major pathogen of this disease, *Treponema denticola*, has several virulence factors, including a major surface protein (Msp) and the surface protease dentilisin. The cytopathic effects of periodontopathic bacteria on epithelial cells disrupt the integrity of the barrier junction, resulting in the inflammation of periodontal tissue. The aim of this study was to investigate the effect of *T. denticola* virulence factors dentilisin and Msp on epithelial cells. The effects of *T. denticola* wild-type, Msp-mutant, and dentilisin-mutant strains on the contact junction in Madin-Darby canine kidney epithelial cells was evaluated based on ohmic values. Cultured oral carcinoma epithelial cells were scratched and exposed to the selected *T. denticola* strains and cell migration determined. Subsequent degradation of adherence proteins and proteins in the contact junctions was evaluated. Dissociation of cell contact junctions was detected in cells infected with wild-type *T. denticola* approximately 30 min after infection, but not in those exposed to the mutants. Inhibition of migration was observed in the wild-type and Msp-deficient mutants. The adherent proteins focal adhesion kinase, ZO-1, and paxillin were hydrolyzed by infection with the wild-type and Msp mutants. These results indicate that *T. denticola* disrupts the function of epithelial cells by hydrolyzing proteins at the intercellular junction and inhibiting healing of epithelial cells *via* hydrolyzed proteins associated with focal adhesion; Msp was also associated with these effects.

Key words: *Treponema denticola* — Periodontitis — Focal adhesion kinase — Paxillin — Epithelial cells

Introduction

Periodontitis is inflammation of the periodontal tissue caused by subgingival microor-

ganisms. The etiologic agent of periodontitis is dysbiosis, which indicates a pathogenic shift in the subgingival microbiome²⁴⁾. One study found that *Porphyromonas gingivalis*, *Treponema*

denticola, and *Tannerella forsythia* showed an increase in lesions associated with severe periodontitis³⁴. An increase in these species was also detected in a recent microbiome analysis using 16S ribosomal RNA sequencing^{1,13} and is thought to play an important role in the process of dysbiosis. *Treponema denticola*, a major pathogen of chronic periodontitis, possesses several virulence factors, such as the protease dentilisin¹⁹ and a major surface protein (Msp)¹¹. Dentilisin shows cytopathic activity against epithelial cells³⁸, adherence to fibrinogen⁴, and degradation of cytokines²⁷, and Msp also exhibits cytopathic activity¹⁰. These activities play an important role in the progression of periodontitis.

The epithelium of the gingival crevice is exposed to the subgingival microbiome. The epithelial cells protect against attacks from periodontopathic bacteria, and the loss of cell integrity allows the invasion of microorganisms into the gingival tissue, resulting in periodontitis. Periodontopathogens, such as *P. gingivalis*, invade epithelial cells and degrade protein-organizing tight junctions by gingipain¹⁴. *Treponema denticola* has been reported to hydrolyze ZO-1 and disrupt junctions between epithelial cells^{7,22}. Although *T. denticola* is known to retard epithelial cells¹⁸, the involvement of its virulence factors, such as that of dentilisin and Msp, in the effect of *T. denticola* infection on the epithelial cell barrier is still unclear. The aim of the present study was to elucidate the involvement of dentilisin and Msp in the effect of *T. denticola* infection on epithelial cells and investigate the mechanism of pathogenicity of this microorganism.

Materials and Methods

1. Bacterial strains and culture conditions

Treponema denticola wild-type strain ATCC 35405⁶, Msp-deficient mutant DMSP3³⁰, and dentilisin-deficient mutant K1²⁰ were maintained in tryptone-yeast extract-gelatin-volatile fatty acids-serum medium²⁸ under anaerobic conditions (10% CO₂, 10% H₂, 80% N₂) as

described previously¹⁹. Gene inactivation was carried out by interruption of the target gene with an erythromycin cassette in the Msp- and dentilisin-deficient mutants, and 40 µg/ml erythromycin was added to the medium to maintain the cassette in their genomes. Microbial cells around the mid-log phase (OD₆₆₀ = 0.4–0.6) were used for infection of epithelial cells with *T. denticola*.

2. Cell cultures

To determine the effect of *T. denticola* infection on tight junctions, Madin-Darby canine kidney epithelial (MDCK) cells NBL-2, which are widely used in epithelial models to investigate tight junctions^{21,26,29}, were used. The MDCK cells were obtained from the Health Research Resources Bank (Osaka, Japan) and maintained in α -minimum essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) (Gibco Life Technologies, New York, NY, USA) and antibiotics at 37°C in a humidified 5% CO₂ atmosphere. Human oral carcinoma epithelial cell line Ca9-22 was obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). These cells were cultured in α -MEM containing 10% FBS and penicillin/streptomycin at 37°C in 5% CO₂ humidified air.

3. Measurement of intercellular junction integrity by transepithelial electrical resistance

Madin-Darby canine kidney cells were used to investigate intercellular junctional disruption as they are normally characterized by tight junctions. These data were obtained by measuring transepithelial electrical resistance (TEER). Monolayers of MDCK cells (5×10^3) were seeded onto BD Falcon cell culture inserts containing 3.0-µm pores in a 24-well configuration (BD, Franklin lakes, NJ, USA). The cells were incubated until they reached confluence. Cell monolayers were used for experiments after 5 days of culture. Apical sites on the MDCK cells were infected with *T. denticola* at a multiplicity of infection (MOI) of 100. The control group was not infected with *T. denticola*. Transepithelial electrical

resistance was measured using chopstick electrodes provided with the TEER Measuring System (Kanto Chemical Co., Inc., Tokyo, Japan).

4. *In vitro* wound healing assay

The effects of *T. denticola* infection on epithelial cell migration were investigated as described previously¹⁷⁾. Briefly, Ca9-22 cells were seeded at a density of 1×10^4 cells/cm² in a culture medium without antimicrobial agents. The cells were cultured until confluent. The cell layers were then scratched using a plastic tip and washed 3 times with fresh culture medium. Next, the cells were incubated with *T. denticola* at an MOI of 100 in α -MEM containing 10% serum in 5% CO₂. In the control group, the cells were treated similarly, but without infection. The closure rate for each scratched area was photographed at each time point and measured using ImageJ/Fiji software (National Institutes of Health [NIH], Bethesda, MD, USA)³¹⁾, and the area of recovery owing to migrated cells calculated. The calculated data were analyzed in triplicate under the same conditions. The results were evaluated with a one-way analysis of variance, followed by Tukey's multiple comparisons test at a 5% level of significance using Prism 9.0 (GraphPad software, Inc., San Diego, CA, USA). All obtained values were expressed as the minimum and maximum ranges, and the mean.

5. Degradation of proteins involved in adherence and intercellular junctions

Localization of proteins involved in adherence and the spreading process of the cells and intercellular junctions was investigated by confocal laser scanning microscopy (CLSM) and western blotting. For CLSM, Ca9-22 cells with or without *T. denticola* infection were treated as described previously²³⁾. Briefly, confluent cells were washed with phosphate buffered saline (PBS) and treated with 4% neutral paraformaldehyde for 30 min and 1% Triton X-100 for 5 min. The cells were then dipped in blocking buffer with 0.5% bovine serum albumin and stained with the following pri-

mary antibodies: rabbit anti-human focal adhesion kinase (FAK) antibody (1: 100 dilution, Abcam, Cambridge, UK); and rabbit anti-human paxillin antibody (1: 100 dilution, Abcam). The secondary antibody, Alexa Fluor 546 goat anti-rabbit immunoglobulin G (IgG), was used for both FAK and paxillin antibodies. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Dojindo Laboratories, Tokyo, Japan), and cell filaments were stained with Alexa Fluor 633 phalloidin (Abcam). The cells were then observed using CLSM 880 (Carl Zeiss, Göttingen, Germany) and ZEN software (Carl Zeiss).

The scratched Ca9-22 cells with or without *T. denticola* infection were cultured for 24 hr. In the experimental group, the cells were infected with each strain of *T. denticola*; uninfected cells were used as the control. Cells were washed with PBS and lysed in radioimmunoprecipitation assay buffer containing 1% 100 mM ethylenediaminetetraacetic acid, 1% protease inhibitor cocktail, 0.5% 1 mM phenylmethylsulfonyl fluoride, and 0.5% 100 mM Na₃VO₄⁴⁰⁾. The concentration of the proteins was determined by a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific K. K., Tokyo, Japan) and Nanodrop (Thermo Fisher Scientific K. K.). The cell lysate was incubated with sodium dodecyl sulfate (SDS) sample buffer at 100°C for 5 min. The lysate was then separated by 10% SDS polyacrylamide gel electrophoresis (Cosmo Bio co, Ltd., Tokyo, Japan) and transferred onto polyvinylidene fluoride (PVDF) membranes (Immobilon, Merck, Tokyo, Japan) by the method described by Towbin *et al.*³⁷⁾ using the Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, CA, USA). Blotted membranes were immunostained with the following primary antibodies: rabbit anti-human FAK (1: 1000 dilution); rabbit anti-human paxillin (1: 1000 dilution); anti-human ZO-1 tight junction protein antibody (1:500 dilution, Abcam); and rabbit anti-human beta-actin (Abcam, for internal control). Antibodies bound to proteins immobilized on the PVDF membrane were detected with peroxidase-conjugated anti-rabbit IgG antibodies, fol-

Fig. 1 Transepithelial electrical resistance (TEER) in epithelial cells infected with *T. denticola* strains.

(A) TEER in Madin-Darby canine kidney epithelial (MDCK) cells infected with *T. denticola* strains was measured every 10 min for up to 90 min. Change in TEER was calculated as follows: $\Delta\Omega = [\text{measured value}] - [\text{baseline value}]$. WT: wild-type strain, Δmsp : Msp-deficient mutant, $\Delta prtP$: dentilisin-deficient mutant, Control: noninfected cells. (B) TEER ohmic levels in MDCK cells infected with *T. denticola* ATCC 35405 for up to 900 min.

lowed by 3,3',5,5'-Tetramethylbenzidine membrane peroxidase substrate (KPL, MD, USA). Images were captured using an ImageQuant LAS (Cytiba, Tokyo, Japan).

Results

1. Change in transepithelial electrical resistance

The TEER value obtained on the first measurement was adjusted to zero to allow a comparison to be made between groups in a single graph (Fig. 1A). The graph shows representative examples of each group. The TEER value for the MDCK monolayer cells infected with the *T. denticola* wild-type strain showed no change from that at baseline level for 10 min,

but then decreased rapidly down to a minimum at approximately 20 min. In contrast, the TEER value for the MDCK monolayer cells infected with the dentilisin or Msp mutants showed no change from that at the baseline level. The decrease observed in the TEER value in the cells infected with the wild-type strain recovered after approximately 150 min (Fig. 1B). The same results, from a decrease to a recovery of the initial TEER value, were observed over repeated experiments.

2. Wound healing assay

No cellular pseudopods were observed immediately in any of the groups and were only marginally observed after 3 hr (Fig. 2A). In the cells without infection and in those

Fig. 2 Migration activity in epithelial cells infected with *T. denticola* strains

Confluent cells were scratched with plastic tips and infected with *T. denticola* at multiplicity of infection of 100. Cells infection was maintained in time series up to 72 hr. Cells were photographed, and scratched area (between two lines) was measured to determine recovery. Dotted line indicates closed sites in scratched areas on both sides of cells. Groups were control, WT: wild-type strain Δmsp : Msp-deficient mutant, $\Delta prtP$: dentilisin-deficient mutant. (A) Scratched at 0, 3, or 24 hr. (B) Recovered area indicates percentage of scratched area covered by migrated epithelial cells. Data are shown as average $\pm$ standard error of mean from 3 independent experiments ($n = 10$, * $p < 0.05$, compared with control, $\dagger p < 0.05$ compared with wild-type).

infected with the dentilisin mutant, the scratched area showed recovery after 24 hr. In the cells infected with the wild-type or Msp mutants, migration showed a significant recommencement after 12 hr. The recovery rates were determined as the mean $\pm$ standard deviation. The recovery rate in each group at 12 hr was as follows: control, $58.5 \pm 5.0\%$; wild-type, $44.4 \pm 3.3\%$; Msp mutant, $44 \pm 7.3\%$; and dentilisin mutant, $55.3 \pm 5.0\%$. The wild-type and Msp-deficient strains had similar effects on cell migration. Recovery in the cells infected with the wild-type strain or Msp

mutant was significantly lower than that in the controls after 12 hr. The scratched area had not filled up at 72 hr in the cells infected with the wild-type strain or Msp-deficient mutant. The scratched area showed a 100% recovery with the control and dentilisin mutants, but not with the wild-type ($54.9 \pm 1.6\%$) or Msp-mutant ($79.0 \pm 9.8\%$) strains. (Fig. 2B). Retardation of cell migration was lower with the Msp-mutant strain than with the wild-type strain.

Fig. 3 Focal adhesion associated proteins in cells infected with *T. denticola*

Ca9-22 cells were exposed to *T. denticola* for 24 hr and observed by immunofluorescence staining. Anti-rabbit focal adhesion kinase (FAK) or paxillin antibodies showed as fluorescent red, nuclei as blue, and cell cytoskeleton phalloidin as cyan. (A): FAK stained in control, (B): FAK stained in wild-type strain infection, (C): Paxillin stained in control and (D): Paxillin stained in wild-type strain infection. In each group picture, results for staining for each protein (red) and nucleus (blue) are shown on left; right side shows merged image of phalloidin staining (cyan) of cytoskeletal filament.

3. Degradation of proteins involved in focal adhesion and intercellular junction

Localization of FAK was observed in both the cytoplasm and at the apex of the pseudopodia as a red color in the control cells (Fig. 3A). Expression of FAK in the cells infected with the *T. denticola* wild-type strain was lower than that in the controls (Fig. 3B). Pseudopod formation showed a decrease in the cells infected with the wild-type strain, and no localization of FAK at the apex pseudopodia was observed.

Paxillin was localized in the cytoplasm, and localization of paxillin at the apex of the pseudopodia was observed in uninfected cells (Fig. 3C). The number of spots representing paxillin in the cytoplasm showed a decrease in the cells infected with *T. denticola* wild-type strain; no localization of paxillin was observed at the apex of pseudopodia (Fig. 3D).

Proteins involved in the adherence and spreading process of the cells and formation of intercellular junctions were determined. These proteins were detected in the cell lysate

at 24 hr after infection by immunoblotting. Degradation of ZO-1 was observed in the cells infected with the wild-type or Msp-mutant strains (Fig. 4A). Degradation of FAK and paxillin was observed in those infected with the *T. denticola* wild-type or Msp-mutant strains, but not with the dentilisin mutant (Fig. 4B, C).

Discussion

The ohmic value of TEER showed a decrease at 30 min after infection with the *T. denticola* wild-type strain, whereas no reduction was observed in the cells infected with the dentilisin or Msp mutants. In earlier studies by this group, a decrease in TEER was observed at 16 hr after infection of the cells with the wild-type strain at an MOI of 10^4 , whereas no reduction was observed in cells infected with the wild-type strain at an MOI of $100^{(2,22)}$. In these earlier studies, *T. denticola* was used in the stationary phase. Therefore, the

Fig. 4 Degradation of proteins involved in adherence and intercellular junctions

Cell lysates of control cells and cells infected with *T. denticola* strains were subjected to western blot analysis. Bands were identified using antibodies against ZO-1 (A), FAK (B), and paxillin (C). For integral protein control, β -actin (D) was used. Each left side was molecular size marker, indicating molecular weight (kDa). Arrowheads of each control lane indicate band of ZO-1, FAK, Paxillin, and β -actin. Estimated molecular mass from immunoblotting of ZO-1, FAK, paxillin, and β -actin was 194 kDa, 119 kDa, 68 and 65 kDa, and 42 kDa, respectively. Subsequent sequential gel order was as follows: control: noninfected cells, WT: wild-type strain Δmsp : Msp-deficient mutant, $\Delta prtP$: dentilisin-deficient mutant.

condition of the *T. denticola* cells may have affected the results. In the present study, a reduction was observed in TEER in epithelial cells infected with the wild-type strain; however, no such reduction was observed in epithelial cells infected with the dentilisin mutant. *Treponema denticola* penetrates the epithelial cell layer²⁵. Another study by the present group investigated how *T. denticola* infected and interacted with epithelial cell rests of Malassez cultures²³. The results revealed that *T. denticola* tended to either

invade cells or establish cell-cell contact. Loss of the intercellular space in epithelial cells infected with *T. denticola* has been observed³⁸. In the present study, ZO-1 in epithelial cells was hydrolyzed by the *T. denticola* wild-type strain but not by the dentilisin-deficient mutant. It has been shown that ZO-1 is a peripheral membrane protein specifically enriched at the points of tight junction membrane contact in polarized epithelial and endothelial cells³⁵. In an earlier study by the present group, ZO-1 was degraded by MDCK cells infected with *T. denticola*²². These results suggest that intercellular junctions are disrupted by dentilisin. Interestingly, in the present study, the decrease in TEER in Msp mutants was smaller than that in the wild-type strain. A previous study by the present group found a slight decrease in expression of dentilisin mRNA in an Msp mutant². In the Msp mutant, the bacterial surface-associated dentilisin levels showed no change, but activity in the culture supernatant decreased. In addition, Msp is involved in adherence to the intercellular matrix^{5,9,39}, and it is possible that the reduction of the adherence and release of cell-free dentilisin affects the disruption of intercellular junctions. These results suggest that both dentilisin and Msp are involved in the penetration of *T. denticola* into the epithelial cell layer *via* intercellular junctions. In the present study, the reduction in TEER showed a recovery at approximately 150 min in cells infected with wild type *T. denticola*. In an earlier study by this group, *T. denticola* was shown to disrupt tight junction protein²². In the present study, the cells were cultured in a humidified 5% CO₂ atmosphere. *Treponema denticola* is a strict anaerobe, which may explain why extracellular *T. denticola* showed a marked decrease over time. Disrupted junctions may recover after a decrease in *T. denticola*, but this remains to be clarified.

In the present study, the migration of epithelial cells was retarded in the wild-type strain and Msp mutant between 12 and 72 hr. The delay observed at 3 hr after infection in cells infected with the wild-type strain was also observed in an earlier study by this group¹⁸.

The retardation of migration observed in the cells infected with the wild-type strain was not seen in the cells infected with the dentilisin mutant, indicating that dentilisin plays a major role in retardation of migration. With the Msp mutant, retardation of migration was attenuated compared with that seen with the wild-type strain. The Msp has the ability to adhere to intercellular matrices, such as fibronectin and collagen^{5,9,39}. These results showed degradation of intercellular contact early after infection with *T. denticola*, suggesting that long-term infection would influence intercellular signaling. It is possible, therefore, that a reduction in adherence affects inhibition of migration.

In the present study, localization of FAK and paxillin in the cytosol of cells infected with the wild-type strain showed a decrease compared with in the uninfected controls. In addition, the localization of these proteins at the pseudopodia disappeared. In the immunoblot, the bands of FAK and paxillin were degraded with the wild-type and Msp mutants. Both FAK and paxillin are focal adhesion-associated proteins. Integrin signaling induces the phosphorylation of paxillin by FAK, and the transduced signal plays an important role in cell attachment, spreading, and migration⁸). Fibroblasts from FAK-null mice exhibit a decreased rate of migration and spreading and an increase in the number and size of peripherally localized adhesions¹⁶). Earlier studies by the present group indicated invasion of epithelial cells by *T. denticola*^{18,33}). The findings of the present study indicate that FAK and paxillin are hydrolyzed after infection with *T. denticola*. The preliminary results also indicated that dentilisin hydrolyzed recombinant paxillin and FAK (data not shown). These results suggest that dentilisin from invasive *T. denticola* is involved in degradation of FAK and paxillin. The expression and activation of matrix metalloproteinase-2 (MMP-2) from the periodontium at 3 to 12 days after infection has been reported³). A more recent report showed that the MMP-2 level upregulated by dentilisin triggered TLR2/MyD88 activation within 24 hr¹²). Thus,

a host-derived protease may also be involved in the degradation of FAK and paxillin. Further analysis is required to elucidate the mechanism underlying the degradation of these proteins in epithelial cells infected with *T. denticola*. One study noted attenuation of migration accompanied by degradation of FAK and paxillin in cells infected with *P. gingivalis*¹⁵). The present results indicate the involvement of FAK and paxillin in the attenuation of epithelial cell migration.

In the present study, disruption of tight junctions and a reduction in migration were observed in cells infected with the wild-type *T. denticola*. In periodontitis, epithelial cells in the subgingival crevice are exposed to continuous attack from biofilm bacteria, including *T. denticola*. *Porphyromonas gingivalis* was also reported to invade epithelial cells and disrupt cell junctions in gingival epithelial cells^{17,36}). Epithelial cells play a key role in wound healing³²). Taken together, these results suggest that *T. denticola* inhibits the wound healing process by disruption of cell junctions and suppression of migration. Further *in vivo* analysis using an animal model is required to clarify this phenomenon.

The present study demonstrated that infection of epithelial cells with *T. denticola* resulted in disruption of intercellular junctions, affecting cell migration. The disruption of intercellular junctions suggested the penetration of *T. denticola* into the gingival tissue. The retardation of migration suggested retardation of wound healing of the epithelial layer in the gingival crevice. Hydrolysis of ZO-1, paxillin, and FAK by dentilisin is suggested to be involved in these phenomena. These effects may contribute to the development of periodontitis during dysbiosis.

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