Journal of the Formosan Medical Association (2014) 113, 549–556

Available online at www.sciencedirect.com

SciVerse ScienceDirect

journal homepage: www.jfma-online.com

ORIGINAL ARTICLE

Association of pocket epithelial cell proliferation in periodontitis with TLR9 expression and inflammatory response

Yen-Chun Chen [a,b], Cheing-Meei Liu [c], Jiiang-Huei Jeng [b,c], Chia-Chi Ku [a,*]

[a] Graduate Institute of Immunology, College of Medicine, National Taiwan University, Taipei, Taiwan
[b] Graduate Institute of Clinical Dentistry, School of Dentistry, National Taiwan University, Taipei, Taiwan
[c] Department of Dentistry, National Taiwan University Hospital, National Taiwan University, Taipei, Taiwan

Received 18 May 2012; received in revised form 29 July 2012; accepted 30 July 2012

KEYWORDS
gingival epithelium;
immunohistochemistry;
matrix metalloproteinase 13;
periodontitis;
Toll-like receptors

Background/Purpose: Inflammatory response is triggered after recognition of microbial ligands by innate receptors such as Toll-like receptors (TLRs) and Nucleotide oligomerization domain (NOD)-like receptors (NLRs). In this study, we examined serial frozen sections of gingival biopsies from patients with gingivitis or periodontitis by immunohistochemical analysis for the topographic expression patterns of selected innate receptors and their association with cell proliferation in clinically healthy and diseased gingival tissues.

Methods: A total of 19 gingival biopsies were collected from patients at the School of Dentistry, National Taiwan University Medical Center according to approved protocol and with informed consent. The specimens were assigned to either the gingivitis group or periodontitis group after clinical evaluation using gingival index. Frozen sections of gingival biopsies were stained with hematoxylin and eosin for histological evaluation. Serial sections of the same samples were stained with a panel of antibodies for immunohistochemical analysis. Expression of each protein marker was compared in the oral versus the sulcular epithelium of the same section.

Results: Expression of cytokeratin 19 (CK19) was markedly increased in the basement membranes of the oral epithelium and in all layers of the pocket epithelium where it caused evident cell proliferation and migration of sulcular epithelial cells into the lamina propria of periodontitis tissue. TLR4 and the cytoplasmic NLRP3 were expressed in all sections examined regardless of disease state. However, expression of TLR9-, CK19- and collagenolytic matrix metalloproteinase-13 and activated NF-κB subunit p65 was more commonly found in periodontitis tissues than in gingivitis tissues.

Conflicts of interest: The authors have no conflicts of interest relevant to this article.

* Corresponding author. R509, Number 1, Section 1, Jen-Ai Road, Taipei, Taiwan.
E-mail address: chiachiku@ntu.edu.tw (C.-C. Ku).

http://dx.doi.org/10.1016/j.jfma.2012.07.043

Conclusion: Activation of TLR9 signaling in the pocket epithelium was highly associated with periodontal inflammation and possibly with loss of tissue integrity. Further studies of mechanisms by which TLR9 signaling is activated in the periodontal epithelium may lead to new strategies for treating periodontitis.

Introduction

Periodontitis is a highly prevalent disease in the general population, with an incidence of about 22% in the West.[1] Based on a recent nationwide, community-based survey in Taiwan, the prevalence of periodontitis in adults aged 35—44 years is about 28.3%.[2] Although risk factors including age, race, hygiene, tobacco use, genetic susceptibility and diabetes have been found to be greatly associated with the development of severe periodontitis,[2–4] chronic inflammation and disruption of periodontal tissues caused by bacterial infection play a primary cause in maintaining the disease progression.[5] Previous studies using immunohistochemical analysis on gingival sections have revealed that all Toll-like receptors (TLRs), except for TLR10, are expressed by the infiltrating immune cells and the gingival epithelium in both healthy tissues and those from patients with periodontitis.[6] However, cells expressing TLR2 and TLR4, which recognize components of the cell wall such as peptidoglycan from Gram-positive and lipopolysaccharide from Gram-negative bacteria, respectively, are selectively increased in the gingival epithelium of periodontitis patients,[7] supporting the notion that bacterial exposure and the development of dental plaque biofilm play important roles in human periodontitis.

In addition to bacterial structural components, unmethylated DNA from periodontopathic bacteria species including *Actinobacillus antinomycetemcomitans* and *Porphyromonas gingivalis* can stimulate TLR9 and induce production of interleukin (IL)-6 and IL-8 from murine macrophages and human gingival fibroblasts.[8] Recent studies have reported that polymorphism in the *TLR9* gene is associated with susceptibility to chronic periodontitis,[9,10] suggesting that TLR9 is also involved in human periodontitis. Periodontal pockets as a result of progressive disruption of the sulcular epithelium (SE) and dental lamina leading to final loss of tooth-supporting alveolar bones are clinical hallmarks of periodontitis. In this study, we examined expression patterns of innate sensor proteins including TLR4, TLR9, and NOD-like receptor (NLR)P3 in the dental epithelium of the gingival tissues from patients with gingivitis or periodontitis by immunohistochemical analysis as well as by analysis of transcriptional levels of pro-inflammatory cytokines by real-time quantitative polymerase chain reaction (q-PCR). We found that while NLRP3 and TLR4 were expressed on the oral epithelium (OE) regardless of the disease state, expression of TLR9 protein in the pocket epithelium was specifically associated with cellular proliferation, nuclear factor (NF)-κB activation and increased expression of pro-inflammatory cytokines as well as collagenolytic matrix metalloproteinases (MMPs) in patients with periodontitis.

Materials and methods

Patient selection and clinical evaluation

Gingival biopsies were collected from the tooth extraction sites in patients with periodontitis ($n = 10$) or from pre-prosthetic surgery sites in patients with gingivitis ($n = 9$) at the School of Dentistry, National Taiwan University Medical Center according to approved protocol (NTUH-REC No.:200705012R) by the Research Ethics Committee of the National Taiwan University Hospital. Signed consent forms and questionnaires of medical history were obtained from volunteer patients after having the study fully explained to them by the investigators. Specimens were immediately embedded in optimal cutting temperature compound (Sakura Finetek, Torrance, CA), snap frozen in liquid nitrogen, and stored at −80°C. Study subjects who had history of diabetes mellitus, major systemic illnesses or smoking, or who had undergone periodontal therapy in the past 6 months were excluded.

The periodontitis group comprised three females and seven males with a mean age of 53.5 years (range, 35—65 years). The gingivitis group had seven females and two males with a mean age of 55.11 years (range, 43—65 years). The criteria for the periodontitis group were an adult patient having at least five sites with probing depths (PDs) $\geq$ 5 mm with accompanying attachment loss $\geq$ 4 mm, and horizontal alveolar bone loss determined by radiography. The criterion for the gingivitis group was an adult patient having a PD $<$4 mm without attachment loss determined by radiography (Table 1). Tissue samples used in the analysis of cytokine expression by real-time q-PCR were also obtained from three periodontitis patients and two healthy individuals without periodontal disease.

Immunohistochemistry

Frozen sections of 5 μm thickness were stained with hematoxylin and eosin for histological evaluation. Serial sections of the same samples were stained with antibody for immunohistochemical analysis. Briefly, frozen sections were fixed in cold acetone (−20°C), washed in phosphate buffered saline (PBS) and treated in 3% H_2O_2 in PBS, followed by nonspecific antigen and biotin blocking (Vector Laboratories, Burlingame, CA). Sections were then incubated at room temperature for 30 minutes with a primary antibody, including: (1) mouse anti-human CK19 (A53-B/A2, mouse IgG2a, Biolegend, San Diego, CA); (2) rabbit anti-human TLR4 (rabbit IgG, Imgenex, San Diego, CA); (3) rabbit anti-human TLR9 (rabbit IgG, Imgenex); (4) goat anti-human NF-κBp65 (goat IgG, Santa Cruz, Santa Cruz,

Table 1 Clinical profile of gingival biopsy sites obtained from patients with gingivitis and periodontitis.

		Gingivitis group ($n = 9$)	Periodontitis group ($n = 10$)	p
Age (y)		55.1 ± 12.6	53.5 ± 9.0	0.89[a]
Sex	Male	1	7	<0.01[a]
	Female	8	3	
Probing depth (mm)		3.13 ± 0.87	6.46 ± 2.47	<0.01[a]
Attachment loss (mm)		3.22 ± 0.67	8.60 ± 3.80	<0.01[a]
Periodontal index[c]		0.58 ± 0.70	1.04 ± 0.80	0.02[b]

Numbers shown are mean ± SD.
[a] Statistically significant differences between the gingivitis and periodontitis groups are compared and calculated by the Student *t* test.
[b] Statistically significant differences between the gingivitis and periodontitis groups are compared and calculated by Fisher's exact test.
[c] Periodontal index from 0 to 3 represents the severity of gingival inflammation.[30]

CA); (5) mouse anti-human MMP-13(VIIIA2, mouse IgG1, Lab Vision, Fremont, CA); and, (6) mouse anti-human NLRP3 (nalpy3-b,mouse IgG1; Abcam, Cambridge, UK). This was followed by incubation with biotinylated secondary antibodies (Vector Laboratories) and horseradish peroxidase (HRP) conjugated streptavidin (Vector Laboratories). Signal was developed with 3, 3′-diaminobenzidine tetrahydrochloride (DAB) chromogen (Vector Laboratories). Incubation with the primary antibody was omitted for secondary antibody controls.

Evaluation of immunostaining

To evaluate the expression of proteins of interest, the gingival epithelium was stratified into the basement membranes, supra-basal and superficial cell layers.[6] Cuboidal/columnar cells with nuclei located at the lower margin of the epithelium were defined as the basement membranes. Above this, cells with spinous/spherical morphology were categorized as the supra-basal layer. The outermost cells with a flattened shape were defined as the superficial layer. Immunostaining results were evaluated and graded by three independent researchers who did not perform the experiments and did not know the disease status of the individual the sample was from. Signal intensity stained with primary antibody was compared with serial sections of the same sample stained with secondary antibody as a negative control. Absence of any signal was graded 0. A positive signal was graded 1 if it was detected only in the basement membranes; if present in the basement membranes and the suprabasal layers, the grade was 2 and if expressed in all layers, the grade was 3. Statistically significant differences in TLR expression patterns in the OE or the SE between patients with gingivitis and periodontitis were determined by the Mann-Whitney rank-sum test.

Real-time q-PCR

Total RNA was extracted from gingival biopsies homogenized in Trizol (Invitrogen, Carlsbad, CA); the first strand of cDNA was synthesized by oligo dT-adaptor primer and Moloney-murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen). Primer sets used to amplify human cytokine genes were as follows: (1) IL-1β:5′-CAC GAT GCA CCT GTA CGA TCA-3′ (sense) and 5′-GTT GCT CCA TAT CCT GTC CCT-3′ (antisense); (2) IL-8: 5′-ACT GAG AGT GAT TGA GAG TGG AC-3′ (sense) and 5′-AAC CCT CTG CAC CCA GTT TTC-3′ (antisense); (3) IL-6: 5′-AAA TTC GGT ACA TCC TCG ACG G-3′ (sense) and 5′-GGA AGG TTC AGG TTG TTT TCT GC-3′ (antisense); and, (4) GAPDH (a human housekeeping gene for control): 5′-ACG GAT TTG GTC GTA TTG GGC-3′ (sense) and 5′-TTG ACG GTG CCA TGG AAT TTG-3′ (antisense). Real-time q-PCR analysis was performed and analyzed by the Microarray and Gene Expression Analysis Core Facility of the National Research Program for Genomic Medicine, Taiwan, using a Bio-Rad iCycler iQTM optical.

Results

Enhanced proliferation of OE in gingival tissues with periodontitis

Gingival tissues are structurally composed of stratified epithelia and the lamina propria, which constitute the major peripheral defense against microbial infections.[11] Although innate immune responses are involved in the severity of periodontal inflammation, the topographic expression of TLRs and its effects on pathological changes in the gingival epithelium remain to be clarified.

To assess the gingival epithelium in different disease states, gingival sections of lesional biopsies from clinically healthy individuals with gingivitis and patients with periodontitis were compared by hematoxylin and eosin analysis (Fig. 1A and B). Cells of the gingival epithelium facing the oral cavity side, defined as the OE, are in close contact and well organized into stratified layers. However, cells facing the tooth side, defined as the SE in healthy tissues, are only in loose contact, resulting in wider intraepithelial spaces and are accompanied by cellular infiltrates beneath the lamina propria (Fig. 1A). Although the histological characteristics of the OE in gingivitis and periodontitis tissues are similar, gingival sections from periodontitis patients revealed a more irregular epithelial—connective interface and migration of gingival SE cells into the lamina propria of the SE (Fig. 1B), which resulted in the characteristic deep periodontal pockets commonly found in periodontitis patients. Therefore, we named the SE in the periodontitis group as the pocket epithelium (PE) to correspond to its disease state.

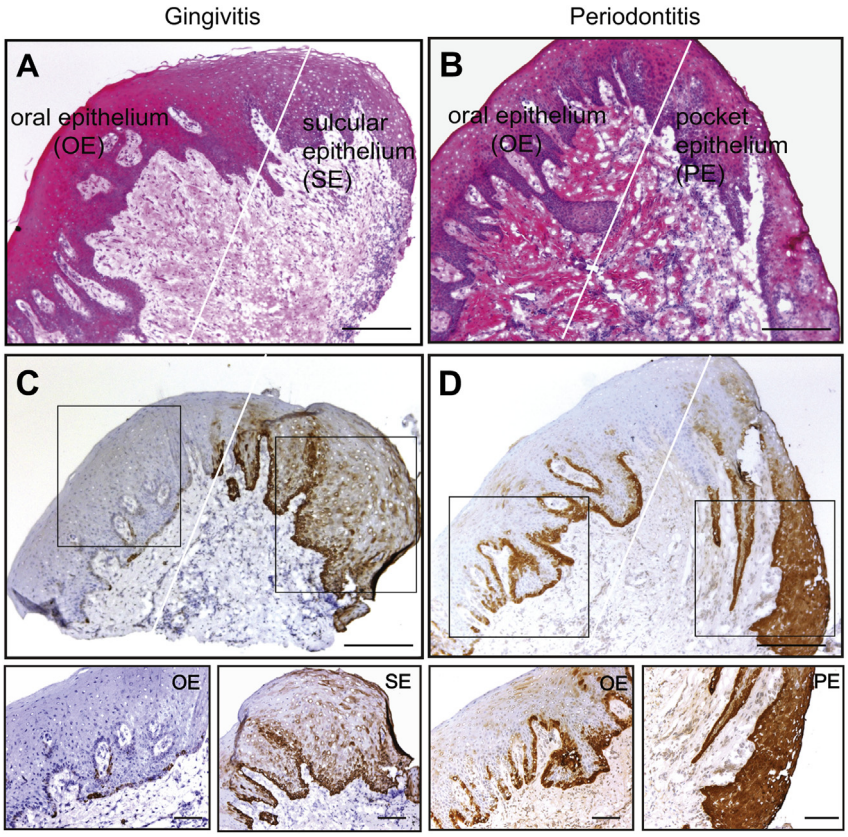

Figure 1 Enhanced migration and proliferation of gingival epithelial cells in periodontitis tissues. Frozen sections of gingival lesion biopsies from patients with (A) gingivitis and (B) periodontitis were stained with hematoxylin and eosin and divided into the OE and the SE in gingivitis tissues or the PE in periodontitis tissues to evaluate the histology and expression of surface antigen, pattern recognition receptors and collagenase throughout the study. Magnification: 100×; scale bar: 50 μm. (C, D) Expression of CK19 in epithelial cells of gingival tissues. Serial sections of the same samples were stained with anti-CK19 antibody and detected with peroxidase substrate DAB (brown). Representative results from one out of nine gingivitis and one out of 10 periodontitis biopsies (magnification: 100×) are shown. The lower panels show higher magnification for the areas in the box (200×). Scale bars: 50 μm. CK = cytokeratin; DAB = 3, 3′-diaminobenzidine tetrahydrochloride; OE = oral epithelial; PE = pocket epithelium; SE = sulcular epithelium.

Expression of cytokeratin (CK)19, a cell proliferation marker,[12,13] was used to assess gingival epithelial cell proliferation in gingival sections from patients in the gingivitis and periodontitis groups by immunohistochemical analysis (Fig. 1C and D). CK19 antigen was predominantly expressed in all supra-basal cells as well as in undifferentiated basement membranes in the SE and the PE from gingivitis and periodontitis groups, individually. However, the intensity of CK19 staining was also significantly increased in the OE of patients with periodontitis but not in those with gingivitis. The enhancement of CK19 expression and the altered topographic distribution of CK19-positive cells in the periodontitis group were highly associated with the breakdown of gingival tissues and periodontal pocket formation, as detected by clinical observation.

Topographic distribution of the expression of TLR4 and TLR9 in the gingival epithelium

Epithelial cells serve as a physical barrier to prevent invasion of periodontal pathogens within the gingival compartment. The extensive proliferation defined by expression of CK19 in gingival epithelial cells within the SE of patients either with gingivitis or periodontitis was consistent with a constant exposure to microbes such as *P. gingivalis*[14] (Fig. 1C and D). When the tissue sections were stained with anti-TLR9 antibody for immunohistochemical analysis, TLR9-positive cells were not evident in the OE and the SE of the gingivitis group (Fig. 2A). However, expression of TLR9 was detected in the basement membranes and the suprabasal layers of the OE and extended to all layers of the PE in patients with periodontitis (Fig. 2B). The differential expression of TLR9 between the OE and the PE from ten periodontitis samples reached statistical significance ($p < 0.001$). In contrast, TLR4 was mainly detected in the basement membranes and the supra-basal layers of the OE but less expression was evident in the superficial para-keratinized layer in gingival sections from both the gingivitis and periodontitis groups (Fig. 2C and D). Expression levels of TLR4 between the SE of the gingivitis group and the PE of the periodontitis group were comparable.

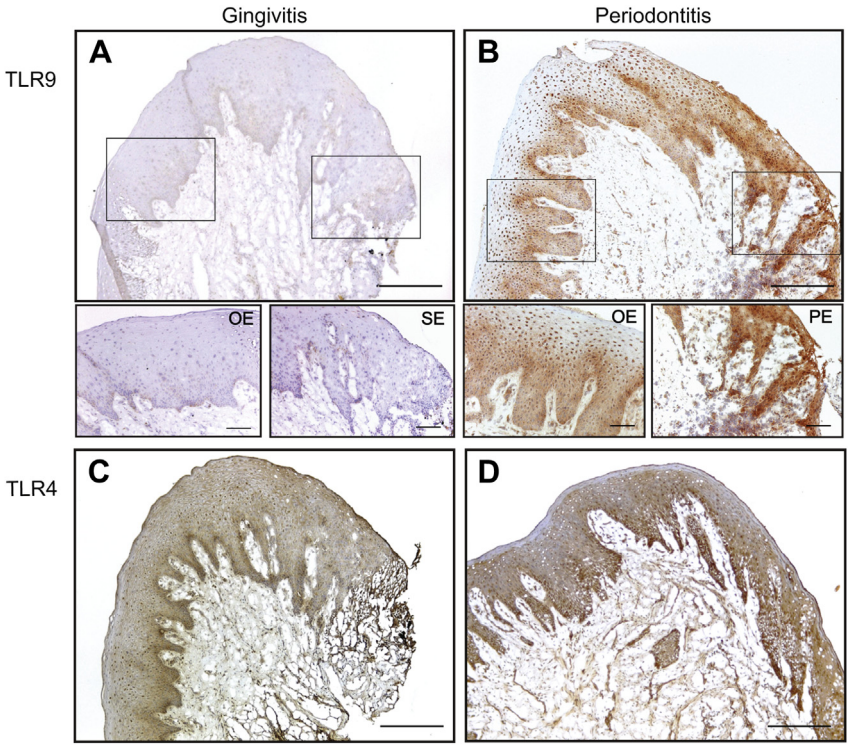

Figure 2 Topographic expression of TLR4 and TLR9 in the gingival epithelium. (A, B) Serial frozen sections of gingival tissues were stained with anti-TLR9 antibody, detected with DAB (brown) and counterstained with hematoxylin. Expression of TLR9 in tissues from patients with gingivitis or periodontitis is shown in the large panels at low magnification (100×) and the expression in the OE, the SE or the PE from the area indicated by the box is shown in the lower panels (magnification: 200×). (C, D) Serial sections of the same samples were stained with anti-TLR4 antibody and detected with peroxidase substrate DAB (magnification: 100×). Shown are representative results from one out of nine gingivitis and one out of ten periodontitis tissues. Scale bars: 50 μm. DAB = 3, 3′-diaminobenzidine tetrahydrochloride; OE = oral epithelium; PE = pocket epithelium; SE = sulcular epithelium; TLR = Toll-like receptor.

NF-κB activation in periodontitis tissues

Binding of bacterial ligands to TLRs activates the NF-κB pathway and results in pro-inflammatory cytokine production.[15] Analysis of NF-κB activation based on translocation of p65 to cell nuclei in gingival sections stained with anti-p65 antibody showed that p65 remained cytoplasmic in the cells of the SE in gingivitis tissues (Fig. 3A). However, p65 was not only extensively expressed in the nuclei of gingival epithelial cells in the OE but also in cells within the PE of patients with periodontitis (Fig. 3B). The results suggested that periodontitis rather than gingivitis was associated with activation of the NF-κB pathway. To assess the levels of pro-inflammatory cytokine expression in gingival tissues, total RNA was extracted from lesional biopsies and converted to cDNA and analyzed by real-time q-PCR for *IL-1β*, *IL-6*, and *IL-8* genes. While the sample size was small in each group (2 in gingivitis group and 3 in periodontitis group), these pro-inflammatory cytokine genes were upregulated in two of three periodontitis lesion tissues but not in those from gingivitis lesions (Fig. 3C).

To examine whether expression of NLRP proteins, which are intracellular innate sensors, was upregulated in gingival epithelium, gingival sections of lesional biopsies from patients with gingivitis and periodontitis were stained with anti-NLRP1 or anti-NLRP3 antibody for immunohistochemical analysis. NLRP1 was not detected in any gingival sections from either gingivitis or periodontitis tissues (data not shown). However, NLRP3 protein was commonly detected in the superficial and suprabasal layers of the OE and extended into the basement membranes of the SE of the gingivitis as well as those of the PE of the periodontitis groups (Fig. 3D and E).

Expression of MMP-13 in periodontitis tissues

Expression of collagenolytic MMPs is reportedly involved in inflamed gingiva affected by periodontitis.[16] Our immunohistochemical analysis of gingival biopsies obtained from patients with gingivitis and periodontitis showed MMP-13 expression was weak or almost undetectable in gingivitis gingiva (Fig. 4A) but strong immunoreactivity of MMP-13 in the OE and the PE of periodontitis tissues (Fig. 4B). Moreover, MMP-13 was detected in some of the basement membranes and the suprabasal layers of the PE in periodontitis tissues.

Discussion

Periodontal pockets are established by irreversible degradation of tooth attachment as a result of proliferation and

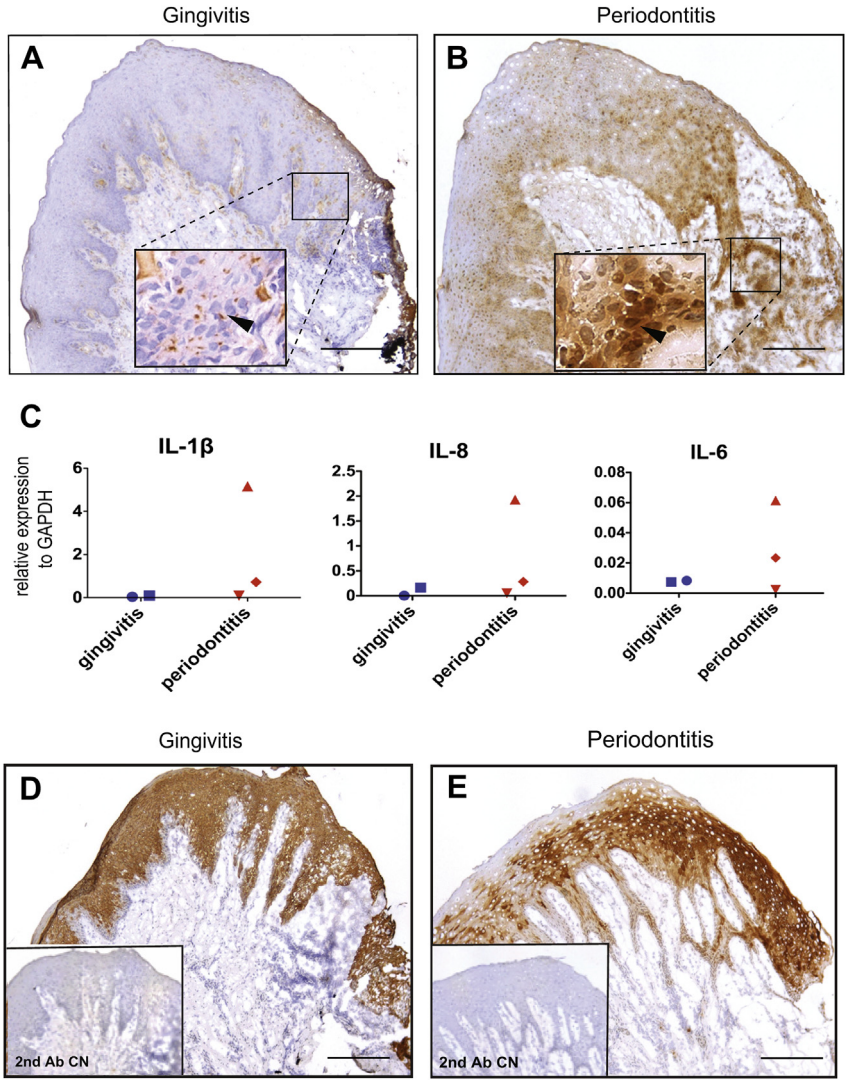

Figure 3 Nuclear translocation of NF-κB subunit p65 and increased proinflammatory cytokine gene expression in periodontitis tissue. Frozen sections of gingival tissues from patients with (A) gingivitis and (B) periodontitis were stained with anti-p65 and detected with DAB (brown). p65 was expressed in the nuclei of the PE in periodontitis tissues but appeared diffused and remained cytoplasmic in gingivitis tissues (magnification: 100×). The inserts show higher magnification for the boxed area (400×); p65 is indicated by arrow heads. Scale bars: 50 μm. (C) Expression of IL-1β, IL-8 and IL-6 mRNA by real-time q-PCR. Total RNA was extracted from periodontitis-affected ($n = 3$) and non-affected ($n = 2$) gingival biopsies. The human housekeeping gene GAPDH was included as an internal control. Data is shown as fold-change relative to GAPDH. (D, E) Serial sections of the same samples were stained with anti-NLRP3 antibody and detected with peroxidase substrate DAB (brown). NLRP3 was expressed by the superficial and supra-basal cells in the OE of gingivitis and periodontitis tissues. Basement membranes either of the SE or of the PE also expressed NLRP3. NLRP3 was not detected in the same section without primary antibody stain (inserts). Shown are representative results from one out of ten periodontitis tissues and one out of nine gingivitis tissues. Magnification: 100×; scale bar: 50 μm. DAB = 3, 3′-diaminobenzidine tetrahydrochloride; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; IL = interleukin; NF = nuclear factor; NLRP3 = NOD-like receptor P3; OE = oral epithelium; PE = pocket epithelium; q-PCR = quantitative polymerase chain reaction; SE = sulcular epithelium.

migration of gingival SE cells.[17] In this study, we examined the topographic expression of innate receptors in periodontitis tissues compared with those affected by gingivitis and demonstrated that expression of *TLR9* by the PE was specifically associated with distinct gingival epithelial cell proliferation and enhanced collagenolytic protein expression in patients with periodontitis.

It is proposed that the homeostasis of periodontium is maintained by rapid cell turnover in the junctional epithelium in response to constant local environmental stimuli, cell damage and repair.[18] A role for enhanced proliferation in the junctional epithelium for promoting the depth of periodontal pockets has also been suggested.[19] We found that CK19 was restricted to the SE in the gingivitis group but was significantly upregulated in both the oral epithelium and the PE of the periodontitis group. These observations were consistent with the appearance of an irregular epithelial–connective interface and migration of

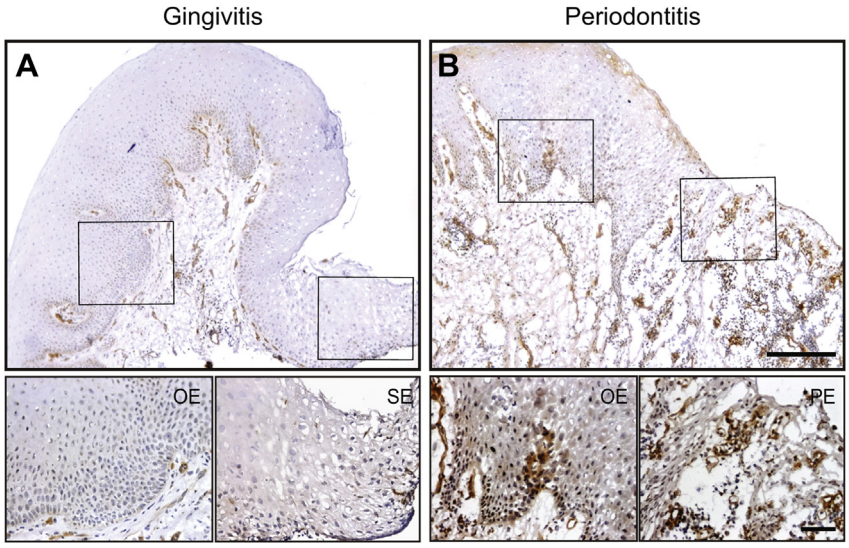

Figure 4 Increased expression of MMP-13 in periodontitis tissues. Serial frozen sections of the samples as described in the legend of Fig. 2 were stained with anti-MMP-13 antibodies and detected with DAB (brown). MMP-13 was expressed in the basement membranes in the OE and the PE in periodontitis tissues but was minimally expressed in gingival epithelial cells of gingivitis tissues (magnification: 100×). The lower panels show MMP-13 expression at higher magnification (200×). Scale bars: 50 μm. DAB = 3,30-diaminobenzidine tetrahydrochloride; MMP = matrix metalloproteinase; OE = oral epithelium; PE = pocket epithelium.

SE cells into the lamina propria of the periodontum as well as the formation of characteristic deep pockets in periodontitis patients by clinical evaluation.

Many bacteria have been found in the oral cavity of humans.[20,21] The epithelial cell—bacterial interactions mediated through TLRs are thought to be important in regulation of tissue integrity through effects on epithelial migration, proliferation and apoptosis.[22] Our analysis of TLR4 and TLR9 expression in clinically healthy and diseased gingival sections showed that while TLR4 is expressed in tissues from patients with gingivitis and periodontitis, expression of TLR9 is associated with periodontitis. Increased TLR9 in cells from the basal to suprabasal layers of OE cells through all cell layers composing the SE in periodontitis-affected tissues has suggested that TLR9 might be activated by bacterial ligands of *A. antinomycetemcomitans* and *P. gingivali*, which are normally not present in intact tissues.[8] It has been reported that invasion of *P. gingivalis* into gingival epithelial cells leads to increased cell proliferation,[23] which in turn may facilitate bacterial penetration into periodontal tissues through the periodontal pocket.[24] Our findings with TLR9 upregulation and NF-κB activation in the pocket epithelium of periodontitis tissues suggested that TLR9 was activated by ligand interactions at the basal—lateral epithelial surface rather than from apical contact.[25] The mechanism by which *TLR9* is activated in the periodontal tissues remains to be explored.

Activation of the NLRP3 inflammasome functions as the second signal for IL-1β maturation and release after TLR activation.[26] Recent studies reported that heat-killed *P. gingivalis* induces IL-1β production from macrophages through the NLRP3-dependent pathway and that a functional NLRP3 inflammasome is present in OE cells.[27—29] In this study, we found that NLRP3 was present in cells within gingival sections from both clinically healthy and diseased tissue samples. This observation suggests that NLRP3 expression could be a host response to constant microbial exposure in the oral cavity and is not a marker to distinguish tissues affected by gingivitis or periodontitis.

MMP-13 is a member of the MMP family of proteins which function to degrade the components of the extracellular matrix and potentially loosen the integrity of gingival epithelium to result in periodontal pockets.[16] MMP-13 was significantly upregulated in periodontitis tissues but was minimally detected in gingivitis tissues. Notably, periodontitis was characterized by overlapping expression of all three innate markers, CK19, TLR9, and MMP-13. Further studies are required to elucidate the cellular and molecular mechanisms by which TLR9 signaling is activated in the periodontal epithelium and may lead to new strategies for treating and preventing periodontitis.

Acknowledgments

This work was supported by grants from National Science Council, Taiwan (NSC97-2320-B-002-005-MY3 and NSC99-3112-B-002-022). We thank the Periodontal Clinic staff at School of Dentistry, NTU Hospital for assistance with patient biopsies, the Microarray and Gene Expression Analysis Core Facility of the National Research Program for Genomic Medicine, Taiwan for real-time q-PCR, Yi-Ping Wang for histological examination, and Ting-Yuan Hu and Min-Yin Ho for assistance with the manuscript.

References

1. Demmer RT, Papapanou PN. Epidemiologic patterns of chronic and aggressive periodontitis. *Periodontol 2000* 2010;**53**:28—44.
2. Wang TT, Chen TH, Wang PE, Lai H, Lo MT, Chen PY, et al. A population-based study on the association between type 2

diabetes and periodontal disease in 12,123 middle-aged Taiwanese (KCIS No. 21). *J Clin Periodontol* 2009;**36**:372—9.
3. Burt B. Position paper: epidemiology of periodontal diseases. *J Periodontol* 2005;**76**:1406—19.
4. Pihlstrom BL, Michalowicz BS, Johnson NW. Periodontal diseases. *Lancet* 2005;**366**:1809—20.
5. Genco RJ. Host responses in periodontal diseases: current concepts. *J Periodontol* 1992;**63**:338—55.
6. Beklen A, Hukkanen M, Richardson R, Konttinen YT. Immunohistochemical localization of Toll-like receptors 1-10 in periodontitis. *Oral Microbiol Immunol* 2008;**23**:425—31.
7. Asai Y, Ohyama Y, Gen K, Ogawa T. Bacterial fimbriae and their peptides activate human gingival epithelial cells through Toll-like receptor 2. *Infect Immun* 2001;**69**:7387—95.
8. Nonnenmacher C, Dalpke A, Zimmermann S, Flores-De-Jacoby L, Mutters R, Heeg K. DNA from periodontopathogenic bacteria is immunostimulatory for mouse and human immune cells. *Infect Immun* 2003;**71**:850—6.
9. Holla LI, Vokurka J, Hrdlickova B, Augustin P, Fassmann A. Association of Toll-like receptor 9 haplotypes with chronic periodontitis in Czech population. *J Clin Periodontol* 2010;**37**: 152—9.
10. Sahingur SE, Xia XJ, Gunsolley J, Schenkein HA, Genco RJ, De Nardin E. Single nucleotide polymorphisms of pattern recognition receptors and chronic periodontitis. *J Periodontal Res* 2011;**46**:184—92.
11. Schroeder HE, Listgarten MA. The gingival tissues: the architecture of periodontal protection. *Periodontol 2000* 1997;**13**: 91—120.
12. Larouche D, Hayward C, Cuffley K, Germain L. Keratin 19 as a stem cell marker *in vivo* and *in vitro*. *Methods Mol Biol* 2005; **289**:103—10.
13. Takeda T, Sugihara K, Hirayama Y, Hirano M, Tanuma JI, Semba I. Immunohistological evaluation of Ki-67, p63, CK19 and p53 expression in oral epithelial dysplasias. *J Oral Pathol Med* 2006;**35**:369—75.
14. Noiri Y, Ozaki K, Nakae H, Matsuo T, Ebisu S. An immunohistochemical study on the localization of *Porphyromonas gingivalis*, *Campylobacter rectus* and *Actinomyces viscosus* in human periodontal pockets. *J Periodontal Res* 1997;**32**:598—607.
15. Akira S, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol* 2004;**4**:499—511.
16. Tervahartiala T, Pirila E, Ceponis A, Maisi P, Salo T, Tuter G, et al. The *in vivo* expression of the collagenolytic matrix metalloproteinases (MMP-2, -8, -13, and -14) and matrilysin (MMP-7) in adult and localized juvenile periodontitis. *J Dent Res* 2000;**79**:1969—77.
17. Page RC, Schroeder HE. Pathogenesis of inflammatory periodontal disease. A summary of current work. *Lab Invest* 1976; **34**:235—49.
18. Genco RJ. Consensus report. Periodontal diseases: pathogenesis and microbial factors. *Ann Periodontol* 1996;**1**:926—32.
19. Takata T, Miyauchi M, Ogawa I, Ito H, Kobayashi J, Nikai H. Reactive change in proliferative activity of the junctional epithelium after topical application of lipopolysaccharide. *J Periodontol* 1997;**68**:531—5.
20. Rudney JD, Chen R. The vital status of human buccal epithelial cells and the bacteria associated with them. *Arch Oral Biol* 2006;**51**:291—8.
21. Thiha K, Takeuchi Y, Umeda M, Huang Y, Ohnishi M, Ishikawa I. Identification of periodontopathic bacteria in gingival tissue of Japanese periodontitis patients. *Oral Microbiol Immunol* 2007; **22**:201—7.
22. Gribar SC, Richardson WM, Sodhi CP, Hackam DJ. No longer an innocent bystander: epithelial toll-like receptor signaling in the development of mucosal inflammation. *Mol Med* 2008;**14**: 645—59.
23. Kuboniwa M, Hasegawa Y, Mao S, Shizukuishi S, Amano A, Lamont RJ, et al. P. gingivalis accelerates gingival epithelial cell progression through the cell cycle. *Microbes Infect* 2008; **10**:122—8.
24. Tribble GD, Lamont RJ. Bacterial invasion of epithelial cells and spreading in periodontal tissue. *Periodontol 2000* 2010;**52**: 68—83.
25. Lee J, Mo JH, Katakura K, Alkalay I, Rucker AN, Liu YT, et al. Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells. *Nat Cell Biol* 2006;**8**: 1327—36.
26. Yu HB, Finlay BB. The caspase-1 inflammasome: A pilot of innate immune responses. *Cell Host Microbe* 2008;**4**:198—208.
27. Kanneganti TD, Lamkanfi M, Kim YG, Chen G, Park JH, Franchi L, et al. Pannexin-1-mediated recognition of bacterial molecules activates the cryopyrin inflammasome independent of Toll-like receptor signaling. *Immunity* 2007;**26**: 433—43.
28. Sugawara Y, Uehara A, Fujimoto Y, Kusumoto S, Fukase K, Shibata K, et al. Toll-like receptors, NOD1, and NOD2 in oral epithelial cells. *J Dent Res* 2006;**85**:524—9.
29. Yilmaz O, Sater AA, Yao L, Koutouzis T, Pettengill M, Ojcius DM. ATP-dependent activation of an inflammasome in primary gingival epithelial cells infected by *Porphyromonas gingivalis*. *Cell Microbiol* 2010;**12**:188—98.
30. Löe H. The Gingival Index, the Plaque Index and the Retention Index systems. *J Periodontol* 1967;**38**:S610—6.