Antigen-specificity of Plasma Cells in Periodontitis Lesions

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ABSTRACT

Periodontitis is characterized by large infiltration of B cells and plasma cells. In this study, $CD19^{+}CD27^{+}CD38^{+}HLA\text{-}DR^{low}$ as periodontitis tissue–plasma cells were identified by flow cytometry. And these cells were also $CD138^{+}$ (a plasma cell marker). In severe periodontitis lesions, large number of $CD138^{+}$ plasma cells which form small clusters disseminated in lamina propria were consistently demonstrated by immunostaining. IgG and IgA-producing plasma cells specific to a key periodontal pathogen, Porphyromonas gingivalis and to a lesser extent against Aggregatibacter actinomycetemcomitans were founded (ELISPOT assay). On the other hand, Ig specific for commensal plaque bacteria-Streptococcus gordonii or self-tissue collagen could not be detected. More studies are required to gain insight into the role of periodontal tissue-plasma cells in protection or pathogenesis of the disease.

บทคัดย่อ

ในรอยโรคปริทันต์อักเสบจะพบบีเซลล์และพลาสมาเซลล์จำนวนมาก การศึกษานี้ใช้วิธีโฟลไซโทเมทรีในการตรวจระบุพลาสมาเซลล์ โดยเซลล์ที่ย้อมติดซีดี19^{+}ซีดี27^{+}ซีดี38^{+}เอชแอลเอ-ดีอาร์$^{ต่ำ}$ถูกระบุเป็นพลาสมาเซลล์ในรอยโรคปริทันต์อักเสบ ซึ่งเซลล์เหล่านี้สามารถตรวจระบุด้วยซีดี138^{+}เช่นกัน การตรวจทางอิมมูโนพยาธิวิทยาโดยการย้อมด้วยซีดี138 ในรอยโรคปริทันต์อักเสบขั้นรุนแรงพบว่ามีพลาสมาเซลล์จำนวนมากกระจายเป็นกลุ่มเล็กๆ ในชั้นลามินาโพรเพรีย เมื่อทำการตรวจหาความจำเพาะต่อแอนติเจนของพลาสมาเซลล์ด้วยวิธีการอีไลสปอตพบว่ามีพลาสมาเซลล์ที่ผลิตอิมมูโนโกลบูลินจีและพลาสมาเซลล์ที่ผลิตอิมมูโนโกลบูลินเอที่จำเพาะต่อแบคทีเรียก่อโรคปริทันต์อักเสบพอร์ไฟโรโมแนส จิงจิวาลิส และมีส่วนน้อยที่จำเพาะต่อเชื้อแอคกรีเกทิแบคเทอร์ แอคทิโนมัยซิเทมโคมิแทนส์ แต่อย่างไรก็ตามไม่พบความจำเพาะของพลาสมาเซลล์ต่อแบคทีเรียประจำถิ่นสเตรปโตคอกคัส กอร์โดไนหรือคอลลาเจนตนเอง ทั้งนี้ต้องมีการศึกษาเพิ่มเติมต่อไป เพื่อให้เข้าใจบทบาทของพลาสมาเซลล์ในโรคปริทันต์อักเสบว่ามีบทบาทในการป้องกันหรือการก่อให้เกิดโรค

Key Words: Periodontal disease, Plasma cells, Antigen-specificity

คำสำคัญ: โรคปริทันต์อักเสบ พลาสมาเซลล์ ความจำเพาะต่อแอนติเจน

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Introduction

One of the most common chronic inflammatory diseases in humans is periodontal disease. The etiology of periodontal disease is microbial plaque biofilm which continuously stimulates local gingival immune response leading to chronic inflammation, loss of connective tissue and bone resorption (Mahanonda, 2012). Moreover genetic and environment could also have an effect on the inflammation process result in periodontium break-down (Kornman, 2008). Inflammatory lymphocyte infiltrates such as B cells and T cells are numerous in lamina propria of periodontal lesion (Brandtzaeg et al., 1965; Lappin et al., 1999; Orima et al., 1999; Page et al., 1976; Seymour et al., 1979a). When the severity of disease is increase, it brings about the transition from gingivitis to perio-dontitis, the lymphocyte infiltration shifts from T cells to B cells and plasma cells (Seymour et al., 1979b). At present the physiological role of B cells and plasma cells in either protection or the pathogenesis of perio-dontitis remain unclear.

B cell response consists of two cellular components; plasma cells and memory B cells. Brandzaeg et al. (1965) described the presence of large number of plasma cell (like morphology) in severe periodontitis tissue under light microscope, therefore suggesting the involvement of immune cells in disease pathogenesis. Later studies using enzymes and surface antigen markers in indirect immunofluorescence and immunohistochemistry confirmed the predominant B cell lesion of periodontitis (Daly et al., 1983; Mackler et al., 1977; Page et al., 1976; Seymour et al., 1979b; Thorbert-Mros et al., 2014; Yamazaki et al., 1993). And these B cells in periodontitis consist mainly plasma cells (Thorbert-Mros et al., 2014).

Antigen-specificity of plasma cells in perio-dontitis tissues was investigated by the technique of enzyme-linked immunosorbent spot (ELISPOT) assay. Major isotype of spot forming cells (SFC) was IgG followed by IgA. (Ogawa et al., 1989a). At present, there is little knowledge about the antigen specificity of antibody that secreted by plasma cells in perio-dontitis lesion.

Objective of the study

In this study, serial cryostat and paraffin-embedded sections were prepared from specimens of severe chronic periodontitis patients in order to investigate localization of plasma cells. Gingival cell extracts from periodontitis sites were used as a source of local tissue plasma cells in order to investigate antigen-specificity against key periodontal pathogen (*Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans)*, commensal bacteria- *Streptococcus gordonii* and type I collagen.

Methodology

Samples

The Human Research Ethics Committee of the Faculty of Dentistry, Chulalongkorn University approved the study protocol (HREC-DCU2014-009) and all subjects signed informed consent before enrollment. Human periodontal tissues were obtained from twelve patients with severe chronic periodontitis and six subjects with clinically healthy periodontal tissues. Each patient had no history of periodontal treatment for the past 6 months.

Severe chronic periodontitis tissues were collected from sites of extracted teeth with hopeless prognosis (gingival inflammation, clinical attachment loss 5 mm or more and severe bone loss 50% of the

root length or more) and not involved other dental diseases such as pulpal lesion. Healthy periodontal tissue samples will be collected from sites with clinically healthy gingiva (no bleeding on probing, probing depth less than 4 mm, no clinical attachment loss and bone loss) during crown lengthening procedure for prosthetic or orthodontic reasons. All subjects had not taken antimicrobial or anti-inflammatory drugs within the previous 3 months. Smokers were excluded. The subjects were recruited from Postgraduate Periodontology Clinic, the Faculty of Dentistry, Chulalongkorn University. The excised tissues were immediately placed in sterile tubes that contain RPMI-1640 medium (Gibco, USA).

Gingival cell preparation

Tissues were washed thoroughly in RPMI-1640 medium and then will be cut into small fragments (1–2 mm^3). These fragments were incubated in RPMI-1640 medium that contained 2 mg/ml of collagenase (Gibco). The ratio of medium plus collagenase to tissues was 1 ml per 100 mg of tissue. After 90 minutes of incubation at 37°C, residual tissue fragments were disaggregated by gentle flushing several times with a pipette, until single cell suspen-sions were obtained. The single cell suspensions were filtered through filter of mesh size 70 µm (BD Biosciences, USA).

Flow cytometric analysis of plasma cells

We previously used flow cytometry to demonstrate the presence of plasma cells in periodontitis tissues which had phenotypic markers of $CD19^+CD27^+CD38^+HLA\text{-}DR^{low}$ (Rattanathammatada, 2013). Extracted gingival cells from severe periodontitis patients were stained with a). anti-human CD19 (FITC), CD27 (PE), CD38 (APC), and HLA-DR (PerCP) monoclonal antibodies (BD Biosciences) or b). anti-human CD19 (FITC), CD27 (PE), CD38 (APC) monoclonal antibodies (BD Biosciences), and CD138 (a well-known marker for plasma cell) (Balakrishnan et al., 2011; Caraux et al., 2010; Hassman et al., 2011; Qian et al., 2010) (PerCP/Cy5.5) monoclonal antibodies (BioLegend, USA) at 4°C for 30 minutes. The stained gingival cells were washed with PBS containing 0.1% albumin and 0.01% sodium azide, and then fixed with 1% paraformaldehyde. Analysis of flow cytometry samples were performed by four-color flow cytometry, FACSCalibur (BD Biosciences).

Immunohistochemistry

The excised periodontal tissues were immediately washed in normal saline solution. For paraffin embedded sections, they were fixed in 10% buffered formalin for a maximum of 24 hours and subsequently embedded in paraffin. Microtome serial 4-micron-thick sections were cut and mounted on glass slides. Sections were deparaffinized. To inhibit endogenous peroxidase, they were incubated with 0.3% hydrogen peroxide solution for 20 minutes. Placing the slides into a 1mM EDTA pH 8.0 and heating for 95°C for 20 minutes for antigen retrieval.

Some tissues were also embedded in the optimum cutting temperature embedding compound, snap-frozen in liquid nitrogen, and stored at -80°C. Serial cryostat section of 4 micron were cut from each specimen, fixed with cold acetone for 10 minutes. To inhibit endogenous peroxidase, they were incubated with 0.3% hydrogen peroxide solution for 20 minutes.

For identifying local plasma cells, single immunohistochemical staining was performed via Polymer/HRP and DAB^+chromagen system (DAKO EnVisionTM G/2 Doublestain System, Denmark) on the sections. They were stained with primary mouse-

anti-human CD138 (plasma cells) (BioLegend) or isotype control. Counterstaining was done with haematoxylin. They were investigated under light microscope.

Enzyme-linked immunosorbent spot (ELISPOT) assay

Multiscreen 96-well filtration plates (Millipore, USA) was coated with a variety of soluble bacterial antigens. These included key periodontal pathogens: a). *Porphyromonas gingivalis,* (*P. gingivalis), b). Aggregatibacter actinomycetemcomitans (A. actinomycetemcomitans)*, c). *commensal plaque bacteria: Streptococcus gordonii (S. gordonii)* (from Professor Fuminobu Yoshimura, Department of Microbiology, School of Dentistry, Aichi Gakuin University, Nagoya, Japan), and d). self-tissue: type I collagen (Sigma-Aldrich, USA) overnight at 4°C in a humidified chamber.

The plate was washed twice with Dulbecco phosphate-bufferd saline (DPBS) and blocked with DPBS containing 10% FBS for 1 hour at 37°C. After washed twice with DPBS, gingival mononuclear cells were added and incubated overnight at 37°C. After that, the plate was washed 3 times with PBS and another 3 times with PBS with 0.05% Tween 20 (PBST). Goat anti human IgG or IgA biotin conjugated (KPL, USA) was added into the plate. After 2 hours of incubation at 37°C, the plate was washed 4 times with PBST and streptavidin-alkaline phosphatase was added to the plate at a 1:1,000 dilution and incubated for 1 hour at 37°C. Then the plate was washed 3 times with PBST and another 3 times with PBS, after that a substrate (5-bromo-4-chloro-3-indolyl phosphate/ nitroblue tetra-zolium; Sigma-Aldrich) was added to allow spots to develop for 5-15 min, and then spots were counted by using an ELISPOT Reader (Cellular Technologies, USA).

Results

$CD19^+CD27^+CD38^+HLA\text{-}DR^{low}$ cells were recognized by anti-CD138 monoclonal antibody

We previously used flow cytometry to demonstrate the presence of plasma cells in periodontitis tissues which had phenotypic markers of $CD19^+CD27^+CD38^+HLA\text{-}DR^{low}$. However, it was not feasible to employ monoclonal antibodies against these 4 surface markers for gingival tissue immunostaining. We, therefore tested if anti-CD138 (Syndecan-1) antibody can recognize gingival plasma cells. Our results in Figure1 clearly showed that all $CD19^+CD27^+CD38^+HLA\text{-}DR^{low}$ plasma cells were positive for CD138, whereas memory B cells ($CD19^+CD27^+CD38^-$) were negative (data not shown).

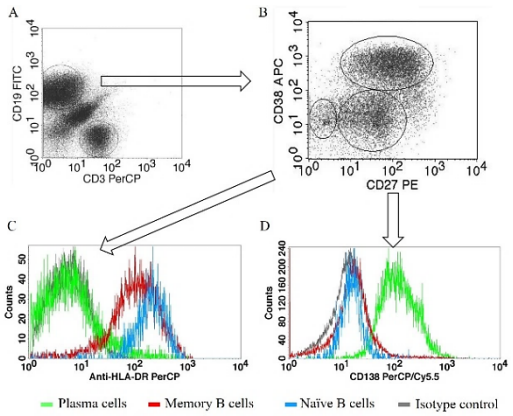

Figure 1 Flow cytometric analysis of plasma cells in periodontitis tissues. Cells extracted from periodontitis tissues were stained with monoclonal antibodies specific to antibody secreting cells ($CD19^+$, $CD27^+$, $CD38^+$). Plasma cells identified as $CD19^+$, $CD27^+$, $CD38^+$, $HLA\text{-}DR^{low}$ cells (C) were also positive for CD138 (D). A representative from three separate experiments.

In periodontal tissues, positive staining of CD138 was observed on plasma cells and epithelium. The small round cells with CD138+ were found in large numbers, forming small clusters disseminating in lamina propria of all severe periodontitis tissues (n =6) but none or very few positive cells was/were observed in clinically healthy gingiva (n=6) (Figure 2A, 2B, 2C, 2D, 2E).

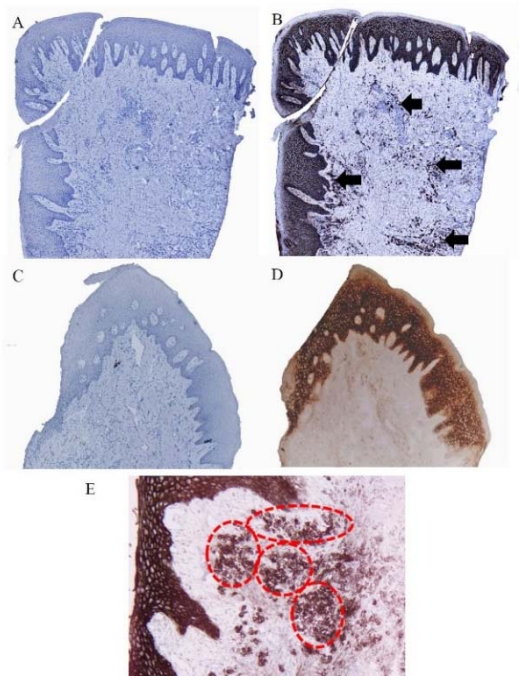

Figure 2 Plasma cell localization in periodontal lesions. Anti-CD138 monoclonal antibody was used to stain plasma cells in periodontitis tissues. Unlike in healthy control tissues (D), CD138 positive plasma cells (arrow indicated) were found in all of the severe periodontitis tissues (B). (A) and (C) showed negative control in healthy and severe periodontitis tissue in order. Magnified photograph demonstrates clusters of CD138 positive plasma cells in lamina propria of periodontitis tissue (E). Magnifications 10x.

Measurement of antigen-specific antibodies by ELISPOT assay

Soluble bacteria antigens from key periodontal pathogens: *P. gingivalis, A. actinomycetemcomitans,* commensal bacteria: *S. gordonii*, and self-tissue antigen: type I collagen were used to assess specificity of antibodies secreted from gingival plasma cells isolated from severe periodontitis patients.

In all 3 patients, we detected gingival plasma cells producing IgG and IgA specific to *P. gingivalis* (Figure3A, B). Mean *P. gingivalis*-specific IgG plasma cells ($1140/10^6$ gingival mononuclear cell [GMC]) was higher than mean *P. gingivalis*-specific IgA plasma cells ($101/10^6$ GMC) (Figure3C). *A. actinomycetem-comitans*-specific IgG plasma cells were found in two patients (Figure3A) but *A. actinomycetemcomitans*-specific IgA plasma cells was not found. We did not detected *S. gordonii*-specific IgG and IgA and type I collagen-specific IgG and IgA plasma cells in all studied periodontitis tissues.

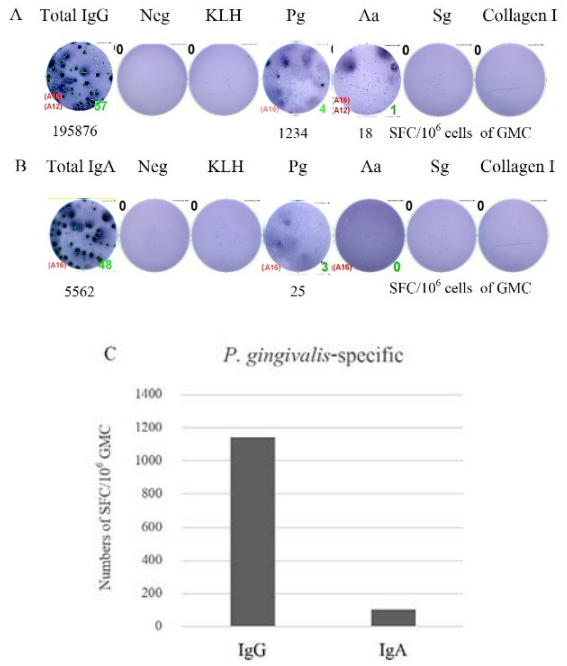

Figure 3 Antigen specificity of plasma cells by ELISPOT assay from one patient: IgG spot forming cell (SFC) (A), IgA SFC (B); Neg: negative, KLH: keyhole limpet haemo-cyanin, Pg: *P. gingivalis*, Aa: *A. actinomycetemcomitans*, Sg: *S. gordonii,* GMC: gingival mononuclear cell. Mean number of SFC per 10^6 GMC: mean *P. gingivalis*-specific IgG was higher than IgA (C).

Discussion and conclusions

Our laboratory recently revisited the role of B cells in periodontitis. We confirmed that the predominant B cells in periodontitis tissue were antibody secreting cells with plasma cell phenotype ($CD19^+CD27^+CD38^+$,$HLA\text{-}DR^{low}$), but not plasma-blasts ($CD19^+CD27^+CD38^+$,$HLD\text{-}DR^{high}$) (Rattana-thammatada, 2013). CD138 has been a well-known marker for plasma cell. In the present study, flow cytometric experiments suggest that extracted gingival cells from periodontitis tissue specimens (n=3) with phenotype of $CD19^+CD27^+CD38^+HLA\text{-}DR^{low}$ also expressed $CD138^+$. We therefore performed immuno-histochemistry staining using monoclonal antibody anti-human CD138 for identifying local plasma cells in periodontitis tissues as compared to those in clinically healthy gingiva.

Data from immunostaining with anti-CD138 monoclonal antibody revealed numerous plasma cells localization in periodontitits lesions that confirms the finding of previous studies (Daly et al., 1983; Mackler et al., 1977; Page et al., 1976; Seymour et al., 1979b; Thorbert-Mros et al., 2014; Yamazaki et al., 1993). Gingival plasma cells appear to form small clusters within lamina propria. There are two possible scena-rios to explain the formation of plasma cells in periodontitis tissues. First, these plasma cells migrate from the closet draining lymph node where B cells are activated and differentiated to plasma cells. The second scenario is based on the previous observations from our group demonstrating the presence of memory B cells in normal gingival tissue. These gingival memory B cells could rapidly activate upon re-encounter with oral bacteria and give rise to plasma cells. Further studies are required to understand the generation and maintenance of plasma cells in periodontitis.

Plasma cells are terminally differentiated B cells that can secrete antibody. Antigen-specificity of plasma cells in periodontal lesions was investigated by ELISPOT assay. Major isotype of spot forming cells was IgG followed by IgA. (Ogawa et al., 1989a). One study reported antigen specificity was fimbriae and lipopolysaccharide of *Porphyromonas gingivalis* (Ogawa et al., 1989b). Besides bacterial etiology, autoimmune reaction has been thought to play role in

periodontal tissue pathology. For example, antibody against collagen was reported in gingival crevicular fluid of periodontitis patients (Sugawara et al., 1992).

Our findings confirm previous studies that plasma cells from periodontitis tissues produce antibody against *P.gingivalis*. We detected higher frequency of *P.gingivalis*-specific IgG plasma cells than *P.gingivalis*-specific IgA plasma cells. Our study extended to investigate the specificity of antibodies to other bacteria antigens and type I collagen, gingival plasma cells from two out of three patients had *A. actinomycetemcomitans*-specific IgG plasma cells, but the frequency was lower when compared to *P.gingivalis*-specific IgG plasma cells. In all studied tissues, we could not measure the presence of plasma cells specific to commensal bacteria: *S. gordonii* and self-tissue antigen: type I collagen.

Periodontitis is a chronic inflammatory disease which contains large numbers of plasma cells in the inflamed gingival tissue. In conclusion, we found that most of these gingival plasma cells produce antibodies against *P. gingivalis* and to a lesser extent to *A. actinomycetemcomitans*. More study is needed to better understand the role of these plasma cells in protection or pathogenesis of the disease.

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