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CXCL13 expression and follicular dendritic cells in relation to B-cell infiltration in periodontal disease tissues

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Background and Objective: B lymphocyte is the dominant infiltrating cell type in periodontitis lesions. CXCL13, produced by follicular dendritic cells, endothelial cells and fibroblasts, is crucial for B-cell trafficking. An association between chronic inflammation and lymphoid organogenesis has been reported in infection and in autoimmune responses, in which T-cell/B-cell follicles with a follicular dendritic cell network are formed. The aim of this study was to examine CXCL13 expression and follicular dendritic cell distribution in relation to B-cell infiltration in chronic inflammatory periodontal lesions.

Material and Methods: Fifty-eight gingival tissue biopsies from patients with periodontitis and 25 samples from subjects with gingivitis were analyzed. Gene expression for CXCL13 and for the CD21 long isoform was analyzed using the reverse transcription–polymerase chain reaction. Immunohistochemical analysis was performed using antibodies to CXCL13, CXCR5, follicular dendritic cells, CD3 and CD19 on serial cryostat sections.

Results: mRNA for CXCL13 was expressed in both periodontitis and gingivitis tissues. The number of $CXCL13^+$ cells was significantly higher in periodontitis than in gingivitis in connective tissues subjacent to the pocket epithelium and positively correlated with the number of $CD19^+$ cells. $CXCL13^+$ cells were distributed in B-cell-dominant areas both with and without follicular dendritic cells. Although obvious reticular networks of follicular dendritic cells were not found in periodontitis and gingivitis, the accumulation of follicular dendritic cells in B-cell-dominant areas in periodontitis was observed in some patients.

Conclusion: These findings suggested that CXCL13 and follicular dendritic cells were involved in B-cell recruitment to, and B-cell distribution in, chronic inflammatory periodontal lesions.

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A chronic inflammatory periodontitis lesion is characterized by lymphocyte infiltration, in which B cells dominate over T cells (1). The distribution pattern of T/B lymphocytes in periodontitis lesions varies, and no specific organization pattern has been identified in association with disease progression. B cells extracted from periodontitis tissues are of a more activated phenotype than those from gingivitis tissues or peripheral blood

(2). Although polyclonal activation of B cells occurs locally (3), the antibody response to the antigens derived from *Porphyromonas gingivalis*, a representative periodontopathic bacterium, was generated in an antigen-specific manner (4).

Recently, it has become apparent that chronic inflammation with lymphoid infiltration in several autoimmune diseases and a few infectious diseases exhibits some characteristics of ectopic lymphoid tissue (5). These include synovitis in rheumatoid arthritis (6,7), stomatitis in Sjögren's syndrome (8,9) and gastritis by *Helicobacter pylori* infection (10,11). Ectopic lymphoid tissue has characteristics such as compartmentalization of B-cell and T-cell populations with a follicular dendritic cell network and the presence of lymphoid-homing chemokines such as CXCL13 (12). The organized structure of the lymphoid tissues is believed to increase the efficiency of antigen presentation and lymphocyte activation in both physiological (12) and pathological (13) conditions.

Chemokine CXCL13 and its receptor CXCR5 play crucial roles in the selective attraction of mature B cells, as well as of a small subset of T cells, to the follicular compartment in the lymph node (14–17). CXCL13/CXCR5 signaling is also implicated in B-cell trafficking into lymph nodes across high endothelial venules (18).

Taken together, we hypothesized that mature B cells are recruited to periodontal lesions by the CXCL13/CXCR5 system, and that ectopic lymphoid-like structures with a follicular dendritic cell network are formed for the production of specific antibodies in periodontitis lesions.

The aim of this study was to examine the expression of CXCL13 and its receptor, CXCR5, and the presence of an ectopic lymphoid-like structure with follicular dendritic cell networks in chronic inflammatory periodontal lesions.

Material and methods

Patients and biopsies

Fifty-eight patients with moderate to advanced chronic periodontitis, referred to the Periodontics and the General Dentistry Clinics of Niigata University Medical and Dental Hospital, took part in this study. Gingival biopsies were obtained at the time of periodontal surgery or extraction of teeth involved with severe periodontitis. As controls, 25 gingivitis tissues showing no destruction of supporting tissue were also obtained from teeth requiring extraction for reasons other than periodontitis (such as orthodontic treatment or pericoronitis). Since clinically healthy gingiva usually displays histological evidence of inflammation similar to that seen in marginal gingivitis, clinically healthy gingiva was grouped as gingivitis. (19,20). The number and clinical status of the biopsy samples used for each experiment are shown in Table 1. Although many of the samples were used for each analysis, several were utilized for different analyses. The experimental protocol was approved by the Institutional Review Board of Niigata University, and informed consent was obtained from all patients prior to inclusion in this study.

Immunohistochemistry

Serial cryostat sections (5 μm in thickness) were cut from 15 periodontitis and eight gingivitis biopsies and stored at −20°C until use, as previously described (1). Sections were stained with mouse monoclonal antibodies specific for CXCL13 (anti-CXCL13; R&D, Minneapolis, MN, USA), CXCR5 (anti-CXCR5; R&D), B lymphocytes (anti-CD19; DAKO, Glostrup, Denmark), T lymphocytes (anti-CD3; DAKO) and follicular dendritic cells (anti-FDC; DAKO). Single or double immunohistochemical staining was performed using an alkaline phosphatase-anti-alkaline phosphatase (APAAP) system (DAKO) and/or an avidin–biotin–complex-immunoperoxidase (ABC-PO) system (Vector, Burlingame, CA, USA), as described previously (1).

Cell analysis

The degree of inflammation was confirmed by hematoxylin and eosin-stained slides. Areas of significant round cell infiltrate in the connective tissues, which contained both T cells and B cells subjacent to the pocket epithelium, as determined on CD3/CD19 double-stained slides, were selected. One or two areas per specimen were selected depending on the degree of cell infiltration. Twenty-two areas from 15 periodontitis specimens, and eight areas from eight gingivitis specimens, were analysed. Positive cells

Table 1. Clinical profile of gingival biopsy sites

	Immunohistochemistry		Real-time RT-PCR analysis of CXCL13		RT-PCR analysis of CD21L	
	Periodontitis (n = 15)	Gingivitis (n = 8)	Periodontitis (n = 20)	Gingivitis (n = 10)	Periodontitis (n = 24)	Gingivitis (n = 16)
Age	53.2 ± 15.0	47.7 ± 20.5	53.9 ± 9.3	29.6 ± 7.0	52.2 ± 12.2	27.3 ± 2.3
Gingival index	1.1 ± 0.9	0.5 ± 0.5	0.7 ± 0.6	0.3 ± 0.5	1.1 ± 0.7	0.3 ± 0.5
Probing depth (mm)	6.4 ± 2.4	3.0 ± 0.9	5.2 ± 1.2	2.2 ± 0.4	5.0 ± 1.5	2.1 ± 0.5
Loss of attachment (mm)	7.6 ± 2.5	3.4 ± 1.6	6.2 ± 2.2	2.2 ± 0.4	5.5 ± 1.9	2.2 ± 0.4
Tooth mobility	2.1 ± 0.8	0.0 ± 0.0	1.3 ± 1.0	0.0 ± 0.0	0.7 ± 0.9	0.0 ± 0.0
Bone loss (%)	75.4 ± 26.3	ND	66.5 ± 25.0	ND	60.5 ± 24.8	ND

Data are expressed as mean ± SD except for bleeding on probing.
CD21L, the CD21 long isoform; ND, not determined; RT-PCR, reverse transcription–polymerase chain reaction.

for CXCL13, CXCR5, CD3 and CD19 were counted for these selected foci using an ocular grid (0.04 mm^2) at a magnification of ×400. The area selected for counting was relocated on the serial sections from each specimen using an ocular grid and histological landmarks.

Real-time reverse transcription–polymerase chain reaction analysis of CXCL13

Total RNA was isolated from gingival tissues obtained from subjects with periodontitis and gingivitis, using TRIZOL (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions, and treated with RNase-free DNase I (Invitrogen). The RNA was then reverse transcribed to cDNA using a random primer (TAKARA SHUZO Co., Ltd, Shiga, Japan) and Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen).

Twenty periodontitis and 10 gingivitis cDNA samples were used for quantitative gene-expression analysis of CXCL13 by real-time polymerase chain reaction using primers and probes for CD19, CXCL13 and glyceraldehyde-3-phosphate dehydrogenase, which were all purchased from Applied Biosystems (Foster City, CA, USA), as described previously (21). Briefly, reactions were conducted in a 25-μL reaction mixture on the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems), following the predeveloped TaqMan assay reagent protocol (Applied Biosystems). The ABI PRISM SDS 2.0 software (Applied Biosystems) was used to analyze the standards and to carry out the quantifications. The relative quantity of each mRNA was normalized to the relative quantity of glyceraldehyde-3-phosphate dehydrogenase.

Gene expression analysis of the CD21 long isoform

Twenty-four periodontitis and 16 gingivitis cDNA samples were analyzed for the presence of gene expression of the CD21 long isoform using the conventional polymerase chain reaction and agarose-gel electrophoresis. The CD21 long isoform was detected by the polymerase chain reaction using primers for the CD21 long isoform (6), and a visible band indicated positivity.

Statistical analysis

The difference in the gene expression of CXCL13 between periodontitis and gingivitis lesions was analysed using the Mann–Whitney *U*-test. Correlation coefficients were analysed between the expression level of CXCL13 and CD19. The statistical significance risk rate was set at $p < 0.05$.

Results

Gene expression analysis of CXCL13

The preliminary experiments carried out using the conventional reverse transcription–polymerase chain reaction revealed that most gingival tissue samples of both periodontitis and gingivitis expressed CXCL13 mRNA. In order to compare the gene expression levels of CXCL13 in periodontitis and gingivitis lesions, quantitative real-time polymerase chain reaction analysis was performed. Although the difference did not reach statistical significance, CXCL13 mRNA expression tended to be higher in periodontitis than in

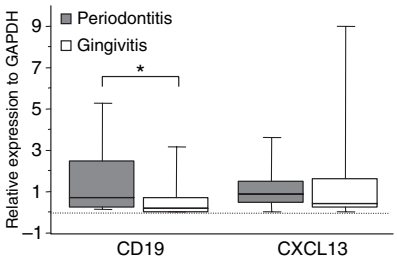

Fig. 1. Comparison of the relative gene expression of CXCL13 and CD19 between periodontitis lesions ($n = 20$) and gingivitis lesions ($n = 10$). The relative quantity of mRNA was normalized to the relative quantity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The box-plots show medians, 25th and 75th percentiles as boxes, and 10th and 90th percentiles as whiskers. *CD19 expression was significantly higher in periodontitis than in gingivitis ($p < 0.05$).

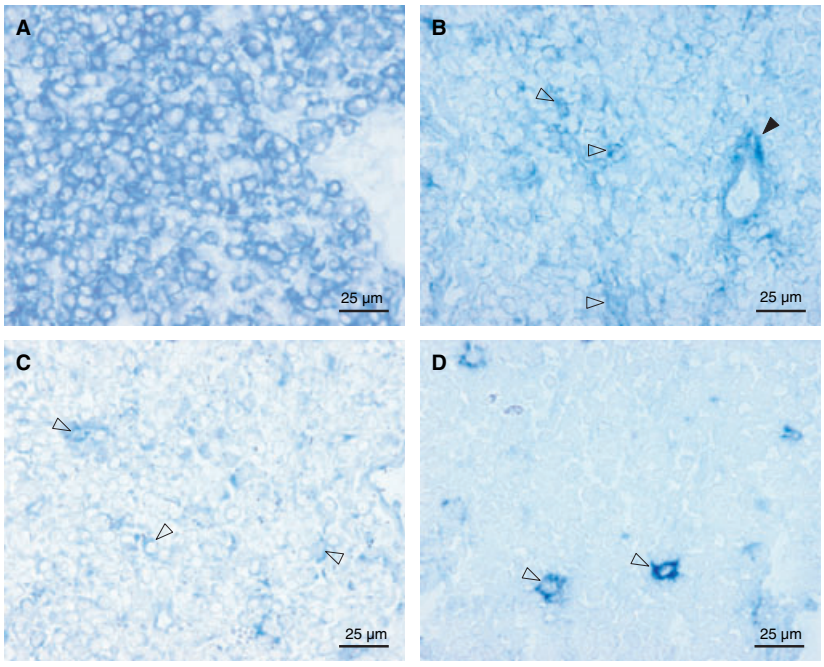

Fig. 2. Immunohistochemical analysis of the connective tissue subjacent to the pocket epithelium of the serial sections of the periodontitis specimen. Serial sections were immunostained for (A) CD19, (B) CXCL13, (C) CXCR5 and (D) follicular dendritic cells. Positive cells were stained blue. Closed arrowheads indicate positively stained endothelial cells. Open arrowheads indicate typical positive cells.

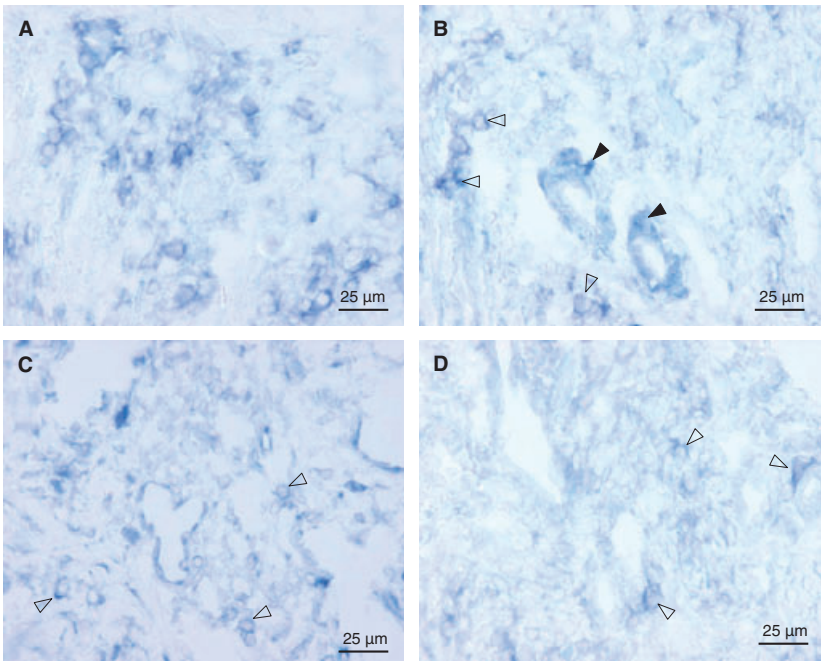

Fig. 3. Immunohistochemical analysis of the connective tissue subjacent to the gingival crevicular epithelium of the serial sections of the gingivitis specimen. Serial sections were immunostained for (A) CD19, (B) CXCL13, (C) CXCR5 and (D) follicular dendritic cells. Positive cells were stained blue. Closed arrowheads indicate positively stained endothelial cells. Open arrowheads indicate typical positive cells.

gingivitis. CD19 expression was significantly higher in periodontitis than in gingivitis (Fig. 1).

Immunohistochemistry of CXCL13 and CXCR5 in inflamed periodontal tissues

The number of $CXCL13^+$ cells was significantly higher in B-cell-dominant periodontitis lesions than in gingivitis lesions (Figs 2, 3, Table 2). The number of $CXCL13^+$ cells correlated with the number of $CD19^+$ cells in periodontitis ($r^2 = 0.334$, $p = 0.0041$) but not in gingivitis ($r^2 = 0.028$, $p = 0.7046$) (Fig. 4). $CXCL13^+$ cells were found as either infiltrating round cells or endothelial cells in some vasculature in both periodontitis and gingivitis (Figs 2B, 3B).

$CXCR5^+$ cells were observed in B-cell infiltrates; however, not all B cells expressed CXCR5 (Figs 2C, 3C).

B-cell aggregates with follicular dendritic cells in periodontitis lesions

In order to elucidate whether B-cell infiltrates in periodontitis were organized like those seen in secondary lymph nodes, the formation of a follicular dendritic cell network was analysed. Although follicular dendritic cells accumulated in the B-cell aggregates, the immunostaining intensity of these follicular dendritic cells was mostly weak. They did not form obvious reticular organization (Fig. 5). On the other hand, scattered follicular dendritic cells with strong immunostaining intensity were found in both periodontitis and gingivitis (Figs 2D, 3D).

Table 2. Number of cells positive for the indicated antigens in the analysed area

	Periodontitis ($n = 22$)	Gingivitis ($n = 8$)
CD3	85.0 ± 25.2	128.0 ± 30.2
CD19	155.4 ± 19.9	34.8 ± 9.2[a]
CXCL13	31.5 ± 6.1	5.3 ± 2.1[a]
CXCR5	25.3 ± 4.0	11.7 ± 3.1
CD19/CD13	6.4 ± 1.6	0.5 ± 0.3[a]
CD3 + CD19	240.5 ± 30.5	162.8 ± 37.2

Data are expressed as mean ± SE.
[a]Significantly different between periodontitis and gingivitis.

Gene expression of the CD21 long isoform in periodontal lesions

The CD21 long isoform is the only definitive marker of follicular dendritic cells (22). mRNA for the CD21 long isoform was detected in 54.2% and 68.8% of periodontitis and gingivitis tissue samples, respectively (Fig. 6). Follicular dendritic cells are one of the major types of CXCL13-producing cells. All samples that demonstrated expression of the CD21 long isoform gene were also positive for expression of the CXCL13 gene.

Discussion

In the present study, we demonstrated that CXCL13 was expressed in association with B cells in periodontal lesions. Immunohistological analysis showed a positive correlation between the expression of CXCL13 and CD19 in periodontitis specimens, suggesting a close association of CXCL13 with B-cell infiltration in periodontal lesions. However, this does not implicate lymphoid organogenesis in periodontitis lesions. Kanemitsu *et al.* demonstrated that CXCL13 is an arrest chemokine for B cells in high endothelial venules and plays an important role in B-cell entry into secondary lymph nodes (23). Strong immunostaining of CXCL13 in endothelium was observed in periodontitis tissues, suggesting that CXCL13 plays a role in B-cell entry to gingival tissues. Follicular dendritic cells are generally believed to be the main source of CXCL13 in normal as well as in inflamed lymphoid tissue. In the present study, $CXCL13^+$ cells were found in B-cell-dominant infiltrate, irrespective of the presence of follicular dendritic cells. Gene expression analysis demonstrated that all of the CD21 long

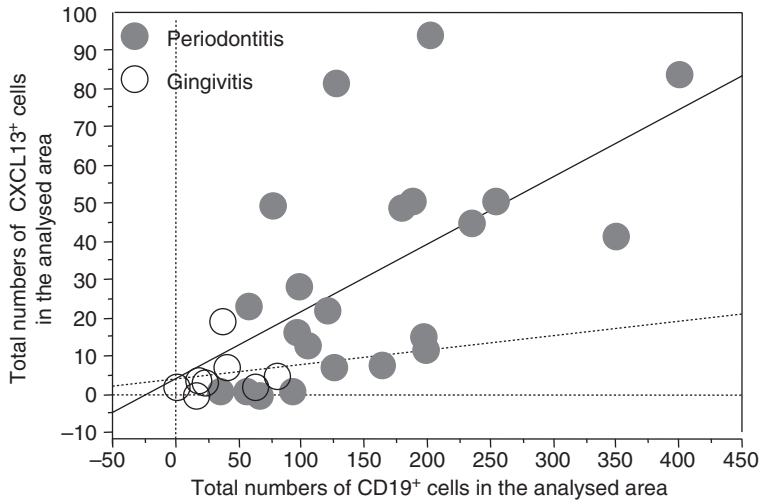

Fig. 4. Correlation of the number of CXCL13$^+$ cells with the number of CD19$^+$ cells in periodontitis and gingivitis tissues. Significantly positive correlations were observed between CXCL13 and CD19 in periodontitis tissues.

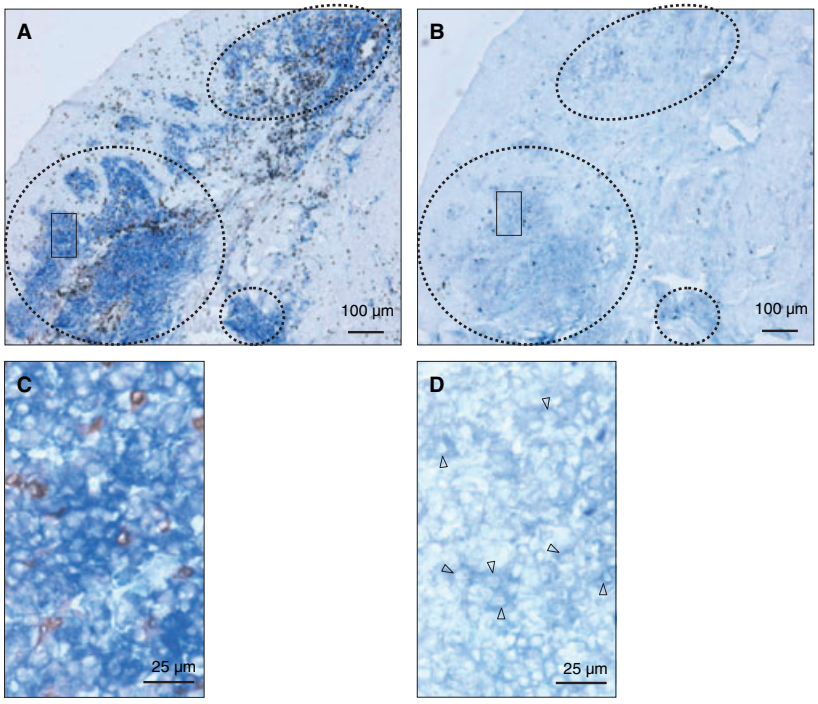

Fig. 5. Serial sections of the periodontitis lesion were stained with (A) anti-CD3 (brown) and anti-CD19 (blue) and (B) anti-FDC (blue). Follicular dendritic cell antigen-positive cells were distributed in accordance with B-cell dominant infiltrate, as shown by the area surrounded with a dotted line. (C) and (D) demonstrate magnified photographs of the insets in (A) and (B), respectively. Open arrowheads indicate typical positive cells.

isoform-positive samples expressed CXCL13 signals. These findings suggest that follicular dendritic cells are a CXCL13-producing cell type; however, other cell types, such as macrophages, may produce CXCL13. In this context, Carlsen *et al.* demonstrated that lipopolysaccharide-stimulated monocytes secreted CXCL13 (24).

CXCL13 and CXCR5 were expressed also in T-cell-dominant gingivitis lesions. These facts suggest that the CXCL13–CXCR5 system could function in the recruitment of some T-cell subsets in inflammatory gingival tissues. Studies by Schaerli *et al.* (16) and Breitfeld *et al.* (25) discussed follicular helper T cells, which were characterized by CXCR5 expression. Follicular helper T cells lack the profile of T helper 1 and T helper 2 cytokines; however, they do exhibit a helper function in B-cell antibody production. The CXCL13–CXCR5 system may involve the recruitment of follicular helper T cells as well as B cells to inflammatory periodontal lesions.

Although follicular dendritic cells were observed in B-cell aggregates of periodontitis lesions, no apparent lymphoid-like structures were found. Even in other B-cell-dominated chronic inflammatory disease, not all lesions demonstrated an ectopic lymphoid structure (6–11), and the association between ectopic lymphoid neogenesis and disease prognosis has not been clarified in those diseases. Generation of the germinal center requires not only mature follicular dendritic cells and CXCL13 but also signallling through CD40/CD40L (26) and CD80, CD86/CD28, inducible costimulatory molecule (ICOS) (27). These molecules were expressed in periodontitis lesions (28); however, regulatory mechanisms for those molecules to organize lymphoid structures remain to be elucidated.

Follicular dendritic cells have important functions in the selection of memory B lymphocytes during germinal center reactions. Follicular dendritic cells present intact antigens to B cells mainly on CD21 (CR2) (20) and CD35 (CR1) (29). The receptor FcγRIIB is also important. The immune complex trapped by FcγRIIB on follicular dendritic cells cross-links the B-cell antigen receptor and the FcγRIIB on B cells. As a result, negative signals through the ITIM motif are minimized in the B cell (30,31). Follicular dendritic cells also block apoptosis in binding B cells (32). Thus, follicular dendritic cells, in addition to T cells, may help B-cell activation in chronic periodontal lesions.

In conclusion, CXCL13 was associated with B-cell recruitment in chronic inflammatory periodontitis lesions. Although periodontitis lesions contain

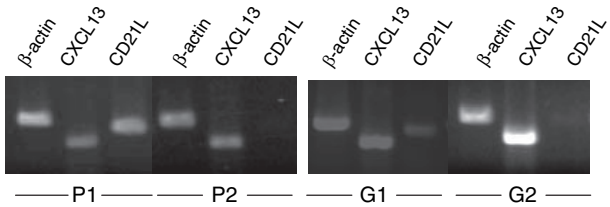

Fig. 6. Gene expression of CXCL13 and the CD21 long isoform (CD21L), a specific marker for follicular dendritic cells in gingival lesions. cDNA was generated from gingival biopsies and amplified with primers specific for CXCL13, the CD21 long isoform and β-actin. Polymerase chain reaction products were visualized after agarose gel electrophoresis. Two sets of representative examples of periodontitis (P1, P2) and gingivitis (G1, G2) are shown. The expression of CD21 long isoform was not dependent on the diseases. Both positive and negative specimens were found in either periodontitis or gingivitis.

follicular dendritic cells, which seemed to be a producer cell of CXCL13, apparent lymphoid organization was not formed. The difference in the roles of follicular dendritic cells and CXCL13 in gingivitis and periodontitis remains to be addressed. Further studies are needed to elucidate the association of B-cell regulation and disease progression in periodontitis.

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