Immunolocalization of interleukin-8 and proliferating cell nuclear antigen in gingival keratinocytes in patients with periodontitis

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The aim of this study was to investigate the relation between local expression of IL-8 and the localization of neutrophilic granulocytes, using CD16 as a marker of neutrophils. We also investigated the correlation between IL-8 and epithelial proliferation using proliferating cell nuclear antigen (PCNA) as a marker of proliferation. The distribution of IL-8, CD16 and PCNA/cyclin was determined by immunocytochemical techniques. We used cryostat-cut sections from gingival biopsies harvested from 5 subjects with and 5 subjects without periodontitis. Our histological examination demonstrated that the localization of neutrophilic granulocytes in gingival tissue from patients with periodontitis did not correlate with the expression of IL-8. In all tissue sections from patients and controls, the inflammatory cells accumulated near the pocket epithelium and only a few leukocytes deviated from this pattern. In the patient group, keratinocytes not belonging to the pocket or junctional epithelium expressed IL-8 without any evidence of a chemoattractant effect on neutrophils. The marker of proliferation, PCNA/cyclin, was expressed in keratinocytes in the basal cell layer. The expression was less pronounced in the control group. Our finding that IL-8 was expressed in proliferating cells suggests that IL-8 may have a role in keratinocyte proliferation. □ *CD16; IL-8; keratinocytes; PC10; periodontitis; proliferating cell nuclear antigen*

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The tissue destruction in periodontitis is mediated by a host inflammatory response to the bacterial activity in the gingival sulcus. The local neutrophilic granulocyte is the first cellular defence against bacterial challenge and more neutrophils can easily be mobilized from the blood stream to the infected area. Studies on conditions with neutrophil defects or deficiency show that these cells are a prerequisite for periodontal health. However, neutrophils have also been found to be an important mediator of tissue destruction (1) in periodontitis (2) and in other human diseases like Crohn's disease, ulcerative colitis, cystic fibrosis, ARDS (Adult Respiratory Distress Syndrome) (3) and psoriasis (4). Several studies suggest local neutrophil hyperactivity with increased release of primary granula content, e.g. β-glucuronidase and elastase (5–7). Among potential inflammatory signal substances involved in periodontitis, interleukin-8 (IL-8) recently has been studied because of its potential importance in the pathogenesis. IL-8 is a chemoattractant for neutrophils and can also, either alone or in concert with other cytokines, activate the cells to respiratory burst and release of proteases (for review, see (8)). IL-8 is produced by various types of cells, including lymphocytes, monocytes, neutrophilic granulocytes, endothelial cells, dermal fibroblasts and keratinocytes, in response to inflammatory mediators such as interleukin-1(IL-1), tumour necrosis factor alpha (TNFα), interferon gamma (IFN-γ) or lipopolysaccharide (LPS) (9, 10). Studies of gingival crevicular fluid have shown conflicting results concerning its IL-8 concentration. However, the total amounts of IL-8 are increased in diseased sites (11, 12) and the total amount has been shown related to clinical parameters (13). Fitzgerald & Kreutzer (14) found that IL-8 was produced by gingival tissues, primarily by epithelial cells, and found that this production was increased at diseased sites. Although much is known concerning immunological mechanisms involved in periodontal disease, the contribution of the gingival keratinocyte to the disease process has not yet been clearly described (for review, see (15)). More studies are available in dermatopathology, where epidermal keratinocytes are thought to play an important regulatory role in the inflammatory response (16–18, for review, see (19)). In psoriasis, overproduction of IL-8 is considered a large part of the disease process and to account for the accumulation of neutrophils that are characteristic of the disease (20, 21).

IL-8 is expressed by epithelial cells during proliferation (22, 23) and has been implicated as a potent mitogen for epidermal keratinocytes (24, 25).

One aim of this study was to investigate the relation between expression of IL-8 and the localization of neutrophil granulocytes. A second aim was to study the correlation between IL-8 and proliferation. A periodontally healthy group was included in the study in order to see if the relations were the same in periodontal health and disease. CD16, an antibody against FCγRIII constitutively expressed on human natural killer cells and neutrophils, was used as a marker of neutrophils. In the present study, we chose proliferating cell nuclear antigen (PCNA/cyclin) as a marker for proliferation. The synthesis of PCNA/cyclin, a nuclear protein, correlates with the

proliferative state of the cell. The rate of synthesis is very low in quiescent cells and increases several-fold shortly before DNA synthesis in cells treated with a variety of mitogenic agents (26–28).

Materials and methods

Gingival biopsies and tissue processing

Gingival biopsies (one per person) were harvested from buccal or lingual sites on molars of 5 patients with periodontitis and from corresponding sites from 5 volunteers without periodontitis. The group with periodontitis comprised 3 males and 2 females, mean age 55 years (range 45–78 years); the healthy control group comprised 3 males and 2 females, mean age 53 years (range 39–72 years). The criteria for periodontitis was an adult patient having at least 5 sites with ≥4 mm horizontal alveolar bone loss on radiographs. The site where a biopsy was taken had a pocket depth of at least 5 mm. The criteria for healthy gingival tissue was an adult patient who showed no attachment loss on radiographs, i.e., not more than 3 mm between the cemento-enamel junction and the alveolar bone and gingival index 0–1. All participants were generally healthy and were not taking any medication that could influence the oral microflora or the inflammatory response. All subjects gave their informed consent to participate and the study was approved by the Ethics Committee at Huddinge University Hospital. The biopsies were harvested from the patients during periodontal surgery. The chosen sites had persisting pockets (at least 5 mm) 3 months after scaling and root-planing. The control biopsies were sampled in connection with preprosthetic surgery. The biopsies were snap-frozen in liquid nitrogen at −196°C and stored at −70°C pending processing. The biopsies were thawed to −22°C and embedded (Cryomount, Histolab Products AB, Gothenburg, Sweden) before cryostat sectioning. We evaluated 40 sections per biopsy. With pilot studies on additional patients, we found that unfixed sections best preserved IL-8 immunoreactivity. Therefore cryosections were used in this study.

Antibodies

Interleukin-8 was detected with a monoclonal mouse anti-human IL-8, recombinant human IL-8_{77} (endothelial derived) (Genzyme Diagnostics, Cambridge, MA, USA) or with a monoclonal mouse anti-human IL-8, E. Coli-derived rhIL-8 (R& D Systems, Minneapolis, MN, USA).

FCγRIII was detected with monoclonal mouse anti-human Fc gamma receptor III, CD16, clone DJ130c (lot 105, DAKO A/S, Glostrup, Denmark).

PCNA/cyclin was detected with monoclonal mouse anti-PCNA (PC10), derived by fusion of spleen cells from a BALB/c mouse with recombinant PCNA with Sp2/0-Ag14 myeloma cells (lot K226, Scandinavian Diagnostic Services (SDS)/ Santa Cruz Biotechnology Inc. Santa Cruz, CA, USA).

Anti-keratin clone AE1, AE3 (lot 83140360, Boehringer Mannheim, Mannheim, Germany) served as positive control and mouse IgG_1, (DAKO A/S, Glostrup, Denmark) served as negative control.

As secondary antibody, we used biotinylated rabbit anti-mouse, mainly IgG biotinylated with activated biotin containing an aminocaproic acid-spacer (lot 067, DAKO A/S, Glostrup, Denmark).

Immunohistochemistry

The tissues were cryosectioned on the day before antibody labelling and stored at −20°C. In order to orientate the biopsies, four sections per biopsy were stained with haematoxylin and eosin and then serial sections were evaluated for the different antigens. No fixation was used. Before staining, the sections were thawed at room temperature for 10 min, blocked of endogenous peroxidase activity with hydrogen peroxide 3% in phosphate-buffered saline with Tween (PBS, 50 mM sodium phosphate, pH 7.6, 200 mM NaCl and 0.1% Tween 20) for 15 min, rinsed and non-specific binding of IgG was blocked with normal swine serum (NSS, lot 107, DAKO A/S, Glostrup, Denmark) 5% in PBS + Tween. After blocking of the non-specific binding, the primary antibody anti-IL-8 (10 μg/ml), CD16 (13.5 μg/ml), anti-PCNA(PC10) (0.5 μg/ml), positive control (10 μg/ml) or negative control (13.5 μg/ml) was added and incubated at 20°C for 1 h. All antibodies were diluted in NSS 5% in PBS + Tween. The sections were rinsed again and overlaid with the secondary antibody for 45 min. The immunoperoxidase labeling was performed using the avidin-biotin-complex (ABC) method (StreptAB-Complex/HRP, lot 087, DAKO A/S, Glostrup, Denmark). The sections were then incubated with the chromogen (3,3′-diaminobenzine tetrahydrochloride 0.05%, DAKO A/S, Glostrup, Denmark) and 0.001% hydrogen peroxide in PBS. The process was stopped with cold tap water and the sections were dehydrated in a graded alcohol series, cleared in xylene and mounted in Pertex (Pertex mounting medium, Histolab Products AB, Gothenburg, Sweden). Sections with the same antibody were incubated with the chromogen for an equally long time. A second series of biopsies used only for PC10 staining was fixed in 4% buffered formalin and embedded in paraffin. Sections (7 μm), cut and mounted on glass slides and air-dried at 56°C overnight, were dewaxed in xylene, placed in decreasing concentrations of alcohol for rehydration and then immersed for 10 min in methanol with 3% hydrogen peroxide. They were then rinsed in PBS + Tween, placed in prewarmed citric acid buffer, pH 6.0 (2 g/l) and incubated for 15 min in a microwave oven set at 65°C. The sections were then cooled down at room temperature for 5 min, after which they were placed in water and immunostaining was performed, as described above.

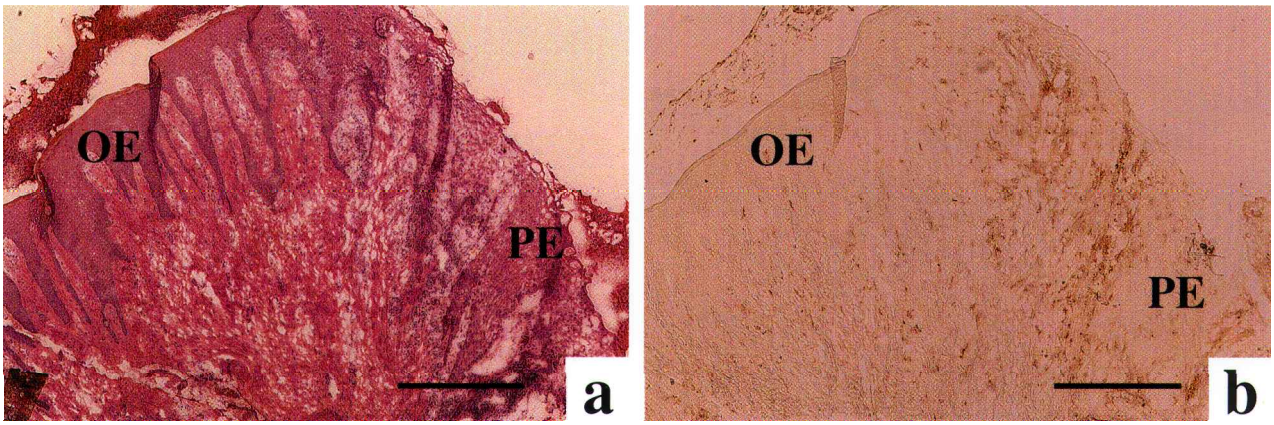

Fig. 1. Serial sections of human gingival tissue showing the localization of the inflammatory infiltrate in a patient with periodontitis. (a) The inflammatory cells, mainly consisting of neutrophilic granulocytes, can be seen nearby and inside the pocket epithelium (haematoxylin and eosin stain) (bar, 500 μm). (b) The IgG Fc receptor on neutrophils (brownish stain) is visualized by labeling with antibody CD16 (bar, 500 μm). OE = oral epithelium, PE = pocket epithelium.

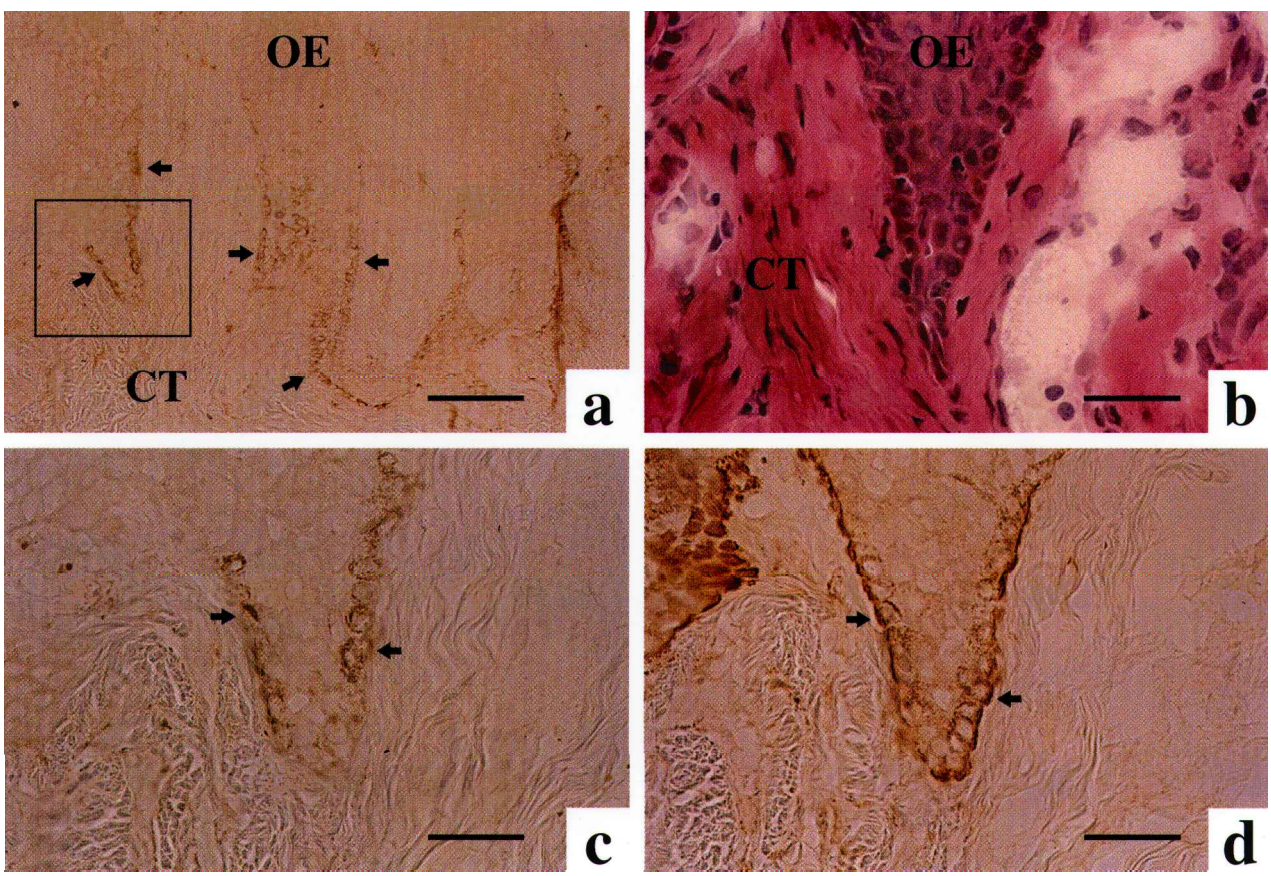

Fig. 2. (a) Section from a patient with periodontitis labeled with anti-IL-8 antibody. IL-8 is present in the basal epithelial cell layer of the oral epithelium (arrows). High power magnifications of the delineated area in Fig. 3a are shown in Fig. 3b–d. (b) Single oral epithelial rete peg. The connective tissue in this area consists mainly of fibroblasts and differs from the connective tissue near the pocket epithelium, which is infiltrated by neutrophilic granulocytes (bar, 50 μm). (c) Keratinocytes of the basal epithelial cell layer labeled with anti-IL-8 antibody (arrows). Note that neutrophils are absent in the connective tissue (bar, 50 μm). (d) Basal epithelial keratinocytes, labeled with the antibody PC10 (arrows), reveal the highly proliferating gingival cells during inflammation and the simultaneous expression of IL-8, as shown in Fig. 3c (bar, 50 μm). OE = epithelium, CT = connective tissue.

Results

Localization of CD16-positive cells

The histological evaluation of gingival inflammation showed an inflammatory infiltrate in all tissue sections (Fig. 1a). The staining pattern of the low-affinity IgG Fc receptor (Fc gamma RIII, CD16 molecule) was constant and similar in all patients with periodontitis. Numerous CD16-positive cells had accumulated near or in the pocket epithelium (Fig. 1b). A few CD16-positive cells could be seen close to the basal membrane of the oral epithelium. The same morphological pattern was present in healthy controls, but with fewer inflammatory cells.

Immunolocalization of IL-8 in gingival epithelium and connective tissue

In the biopsies from patients with periodontitis, intercellular or membrane-associated immunoreactivity for IL-8 was weak but detectable in the keratinocytes of the basal cell layer in the oral and pocket epithelium (Figs 2a, c). An irregular but clearly detectable staining of IL-8 was seen in the keratinocytes of the basal epithelial cell layer in the oral and pocket epithelium. Diffuse staining of IL-8 could also be seen in some neutrophils adjacent to the epithelial basal cell layer in the connective tissue, or in migrating neutrophils near or in the pocket epithelium. The immunolocalization of IL-8 varied between patients and in one patient no staining could be detected. The reason why IL-8 could not be detected in this patient is unclear. However, there was no clinical difference that could explain this. The two different monoclonal anti-IL-8 antibodies used in this study showed the same staining. No staining was seen in the negative or healthy controls.

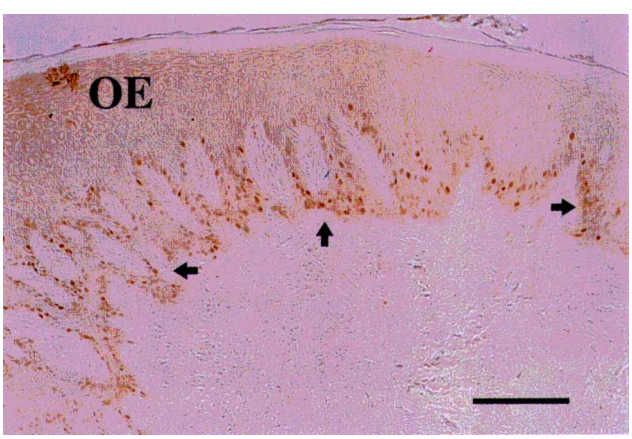

Fig. 3. Proliferating cell nuclear antigen (PCNA/cyclin), detected as PC10 in a fixed paraffin-embedded section of a healthy control (gingivitis), reveals proliferating cells in the basal epithelial cell layer (arrows) (bar, 100 μm). OE = oral epithelium.

Immunolocalization of proliferating cell nuclear antigen

The gingival sections from patients with periodontitis revealed high amounts of the proliferating cell nuclear antigen (PCNA/cyclin), when stained with the anti-PCNA/cyclin (PC10) antibody. Staining intensity and number of positively stained cells did not obviously differ between sections from the various patients and controls, respectively. The protein detection was exclusively seen only in the keratinocytes of the basal cell layer in the oral and pocket epithelium. The healthy controls also showed proliferating cell nuclear antigen (PCNA/cyclin), when stained with the anti-PCNA/cyclin (PC10) antibody, but the number of positive cells was obviously lower. No staining was evident on other cells. PCNA and IL-8 were both expressed in the basal layer of oral and pocket epithelium (Figs 2c, d). However, there were no signs of CD16 expression or infiltration by any other inflammatory cells close to the oral epithelium (Fig. 2b). In a preliminary study we used paraffin-embedded sections for PC10 staining and found that the staining was confined almost entirely to the nucleus. Fewer cells expressing the protein were detected, but the signal was still exclusively in the keratinocytes in the basal cell layer (Fig. 3).

Discussion

The present study shows that IL-8 is expressed by gingival keratinocytes in chronically inflamed gingiva but not periodontally healthy. The localization of neutrophilic granulocytes in gingival tissue from patients with periodontitis does not correlate with the local expression of interleukin-8 in oral epithelium. We found a very distinct localization pattern of the neutrophils in the gingival tissue towards the pocket. In all tissue sections from patients and healthy controls, the inflammatory cells accumulated near the pocket epithelium and only a few leukocytes deviated from this pattern. On the other hand, in the patient group, labeling with anti-IL-8 also reveals that keratinocytes not belonging to the pocket or junctional epithelium express IL-8 without any evidence of a chemoattractant effect on neutrophils. Whether this is because no IL-8 is released extracellularly from keratinocytes expressing the cytokine together with proliferation alone, in other words, in a region that is not inflamed, or whether some additional stimuli are needed to induce neutrophil migration cannot be shown with our study design. A similar pattern could not be revealed in the control group, since we failed to detect IL-8 in the sections from this group.

In the present study, expression of PC10 was not confined to the nucleus but could be seen in the entire cell. This is probably due to the fact that we used cryo-sections and not paraffin-embedded sections. The variations in the signal of PC10 in cryosections and paraffin-embedded sections could be due to differences in the preparations of tissues before staining. This accords with the report by Hall et al. (29). IL-8 and proliferating cell nuclear antigen

are both localized in the basal layer of gingival keratinocytes. This supports the view that IL-8 acts as an autocrine mitogen in keratinocytes. Several studies have shown that IL-8 is both a potent chemoattractant and a mitogen for epidermal keratinocytes (24, 25, 30, 31), which might also be true of gingival keratinocytes. Some authors have found that epidermal keratinocytes in vitro (22) and in vivo (23) release IL-8 during proliferation. Epidermal keratinocytes express IL-8 receptors (32) but it is not known whether there are IL-8 receptors on gingival keratinocytes. If this were the case, it suggests an autocrine IL-8 effect on oral keratinocytes as well. The view that IL-8 functions as a growth factor for keratinocytes accords well with the epithelial proliferation involving the formation of characteristic rete pegs seen in periodontitis. In periodontitis, such pegs may be caused by highly proliferating oral keratinocytes in response to inflammatory mediators. The formation of more prominent rete pegs to increase the tissue surface area seems logical because they provide a major route for metabolic exchange between the epithelium and connective tissue.

Local expression of IL-8 in gingival epithelial cells has been reported by several authors. Tonetti et al. (33) showed a preferential localization of IL-8 expression in the superficial layers of the junctional epithelium. Gainet et al. (34) found that all epithelial cells in the gingiva express IL-8 mRNA, while Fitzgerald & Kreutzer (14) observed that anti-interleukin-8 antibody could be found only in the epithelial layer of gingival tissue. However, within the epithelium, no binding of anti-IL-8 was present in the most apical layers of the cells or in the outermost epithelial cell layer, but binding was particularly strong in the rest of the epithelium. Methodological differences may account for the disparate findings, but all studies show that the local expression of IL-8 is confined to gingival keratinocytes. It is possible that IL-8 and its inducing mediators, e.g. IL-1 and TNF-α (18), may be involved in the recruitment and proliferation of epithelial cells (30) and that local production of IL-1 and TNF-α can induce surrounding cells to produce IL-8 in a cascade phenomenon (34). Tuschil et al. (30) showed that IL-8 enhanced the proliferation in keratinocytes by 10% to 40%, levels comparable to those observed with epidermal growth factor (EGF). Although local production of the cytokine in inflamed sites is undoubtedly responsible, at least in part, for cutaneous trafficking and activation of inflammatory cells, the role of IL-8 as an autocrine or paracrine growth factor regulating the characteristic keratinocyte hyperproliferation seen in skin diseases such as psoriasis is as yet not understood. This is also unclear in periodontitis, but locally produced IL-8 may be an important factor in maintaining the hyperproliferation of keratinocytes (30) seen in both psoriasis and periodontitis. Since epithelial downgrowth along the root surfaces is a characteristic of periodontitis (35), our finding of an association between IL-8 and keratinocyte proliferation may be of importance. This descriptive study indicates that the expression of both IL-8 and PCNA/cyclin is lower in the periodontally healthy control than in the patient with periodontitis. However, in order to do a conclusive comparison between patients and controls, it would be necessary to do an objective quantification of the samples. Clearly, more research is necessary to explore the contributions of IL-8 in the pathogenesis of periodontitis and to correlate the expression of IL-8 with cellular functions such as proliferation.

In conclusion, this study shows that IL-8 is immunolocalized, most likely expressed by keratinocytes in the basal epithelium layer, not only in the pocket epithelium but also in the oral epithelium of subjects with clinical signs of attachment loss. No expression of IL-8 could be observed in the samples from the control group. Although IL-8 was immunolocalized in the oral epithelium, neutrophilic granulocyte migration was seen only in the region of the pocket and junctional epithelium.

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