Oral Diseases (2007) 13, 77–81. doi:10.1111/j.1601-0825.2006.01250.x
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ORIGINAL ARTICLE

Location of proliferating gingival cells following toothbrushing stimulation

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OBJECTIVES: Mechanical stimulation by toothbrushing promotes healing of gingivitis through accelerating cell proliferation. Junctional epithelium proliferates at periodontal pocket formation. A question is arisen whether toothbrushing contributes to the repair of gingival inflammation or deterioration of pocket formation. The location of proliferating cells in gingiva stimulated mechanically by toothbrushing was investigated.

MATERIALS AND METHODS: A total of 24 teeth of dogs underwent daily plaque removal with a curette (plaque removal) or both plaque removal and toothbrushing (toothbrushing). Proliferative activity of gingival cells in six individual zones was evaluated by assaying expression of proliferating cell nuclear antigen (PCNA).

RESULTS: Toothbrushing increased densities of PCNA-positive basal cells in the junctional epithelium, connective tissues adjacent to the junctional epithelium, the alveolar bone of the oral epithelial side and the oral epithelium. However, the densities of PCNA-positive cells at the apical portion of the junctional epithelium, connective tissues adjacent to the cementum and the alveolar bone of the periodontal ligament side did not increase following toothbrushing.

CONCLUSIONS: Toothbrushing promotes proliferation of gingival cells other than fibroblasts in periodontium and basal cells in the apical portion of the junctional epithelium. The repair of periodontal tissues might be promoted by toothbrushing within the limit of the direct mechanical stimulation.

Oral Diseases (2007) **13**, 77–81

Keywords: toothbrushing; mechanical stimulation; gingival cells; proliferating cell nuclear antigen; animal studies

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Received 15 July 2005; revised 21 October 2005; accepted 13 January 2006

Introduction

Proliferation of basal cells and fibroblasts plays a major role in maintaining healthy periodontal tissue. A high rate of epithelial turnover, which leads to desquamation, prevents bacterial invasion into periodontal tissue (Listgarten, 1986). Fibroblasts, the most predominant cells in connective tissue, are engaged in production of collagen fibers (Lindhe and Karring, 1997).

On the other hand, basal cell proliferation results in initiation of periodontitis. Increased proliferative activity of basal cells in junctional epithelium (JE) is followed by apical migration of JE in the initial stage of periodontitis (Ekuni *et al*, 2005). Cell proliferation of periodontal tissue is promoted both at formation of periodontal pocket and healing of periodontal inflammation.

Effects of toothbrushing on gingival cell proliferation are not observed more than 0.5 mm from the brushed area (Sakamoto *et al*, 2003). Alveolar bone and teeth may interrupt conduction of mechanical stimulation by toothbrushing to gingiva. These results highlight the location of cell proliferation by toothbrushing in periodontium.

Purpose of the present study was to evaluate the location of proliferating gingival cells in dog gingiva after toothbrushing.

Materials and methods

Animals

Twelve mongrel dogs (mean weight 9.2 kg) were fed a soft diet (Dog-chow DS diet; Oriental Yeast Co., Tokyo, Japan) during the experimental period. The animal experiments were conducted in accordance with institutional guidelines of the Animal Center for Medical Research of Okayama University.

Treatments

At the beginning of the pre-experimental period, supra- and subgingival scaling of all experimental teeth were performed and supragingival plaque was removed once a day with a curette (Hu-Friedy Manufacturing Co. Inc., Chicago, IL, USA) for 2 weeks.

At the beginning of the experimental period, one of each pair of quadrants in each dog was selected for the experimental group. Daily supragingival plaque removal with a curette was performed on a quadrant of each dog (plaque-removal group), and plaque removal plus toothbrushing was performed on another quadrant (toothbrushing group). The buccal tooth surface and gingiva were brushed for 20 s per tooth at a force of 1.96 N, calibrated using a strain gauge (N-11-FA-5-120-11-VSE1; NEC San-ei Instruments, Ltd, Tokyo, Japan) (Tomofuji *et al*, 2003). A plastic stent was attached to the tooth in order to fix the position of the toothbrush. The toothbrush had two rows of nylon bristles, with three tufts per row and 50 filaments (diameter 0.2 mm) per tuft (Horiuchi *et al*, 2002). The treatment was continued for 3 weeks. All procedures were performed under intramuscular sedation with ketamine hydrochloride (20 mg kg^{-1} body weight).

Immunostaining

The dogs were killed with an intravenous injection of sodium thiamylal, and their teeth and gingivae were then fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C overnight, followed by decalcification with a 10% tetrasodium-EDTA aqueous solution (pH 7.4) for 4–8 weeks at 4°C. Three paraffin-embedded buccolingual sections (4 μm) from each tooth were randomly selected for staining.

Proliferating cell nuclear antigen (PCNA) was stained immunohistochemically using the avidin–biotin complex method (Hsu *et al*, 1981). Mouse monoclonal antibody against PCNA (Novacastra Laboratories, Newcastle, UK) was diluted at 1/200 in phosphate-buffered saline. The color was developed with 3-3′-diamino benzidine tetrahydrochloride. Sections were counterstained with Mayer's hematoxylin.

Histometrical analysis

After the specimens were randomly numbered so that the examiner was blinded to the specimen treatment assignment, the following histometrical analyses were performed using a microscope at a magnification of ×400.

Twelve standard areas (0.1 mm) in the basal layer of the JE were defined from the most apical portion to the coronal portion (Figure 1). The numbers of PCNA-positive basal cells and total basal cells were counted in each standard area. Also, PCNA-positive fibroblasts and total fibroblasts were determined in the following connective tissue zones (0.1 mm × 0.1 mm each) (Figure 1): (i) subjacent to the JE [12 serial areas from the cemento-enamel junction (CEJ)]; (ii) adjacent to the cementum (five serial areas from CEJ); (iii) adjacent to the alveolar bone of the periodontal ligament side (five serial areas from the top of the alveolar bone); (iv) adjacent to the alveolar bone of the oral epithelial side (five serial areas from the top of the alveolar bone); and (vi) subjacent to the oral epithelium (three areas at the coronal, middle and apical aspect of free gingiva).

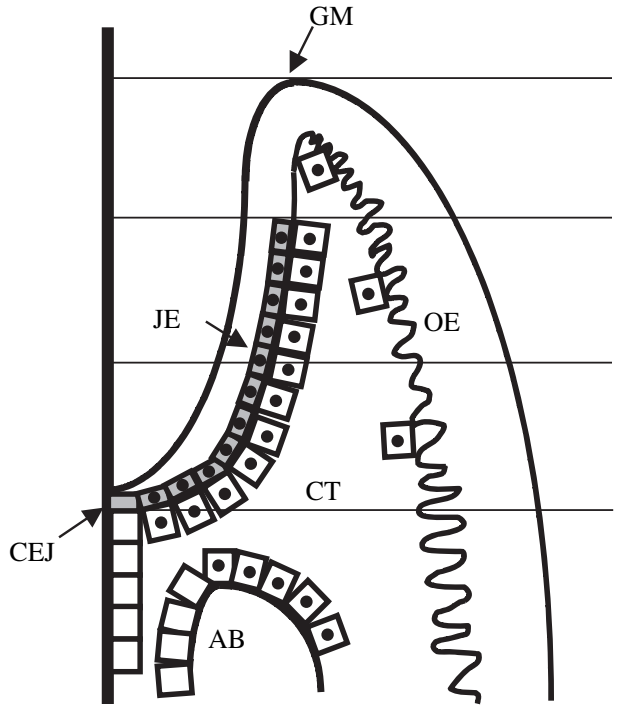

Figure 1 Schematic drawing of the buccolingual sections with histometric parameters and landmarks. GM, gingival margin; JE, junctional epithelium; OE, oral epithelium; CT, connective tissue; CEJ, cemento-enamel junction; AB, alveolar bone; ▭, basal layer area examined (0.1 mm); ▭, connective tissue area examined (0.1 mm × 0.1 mm). ▭ and ▭, significantly proliferating area by toothbrushing

Statistical analysis

Mean values of the histologic data were calculated for each tooth. The Wilcoxon's signed-ranks test was performed to detect statistically significant differences between the groups. All calculations were performed using a statistical software package (SPSS 10.0 J for Windows, SPSS Japan, Tokyo, Japan).

Results

Densities of PCNA-positive cells and total cells in the plaque removal and toothbrushing groups are presented in Table 1 and Figure 2. The density of PCNA-positive basal cells in the toothbrushing group was 1.8 times that of the plaque-removal group. The connective tissues subjacent to JE, adjacent to the alveolar bone of the oral epithelial side and subjacent to the oral epithelium had a significantly higher density of PCNA-positive fibroblasts in the toothbrushing group than in the plaque-removal group. Connective tissue adjacent to the cementum and the alveolar bone of the periodontal ligament side had higher number of fibroblasts than the other three zones in the plaque-removal group.

The PCNA-positive ratios (PCNA-positive cells/total cells) of basal cells and fibroblasts, in relation to the distance from CEJ to the coronal and/or apical sides, are shown in Figures 3 and 4, respectively. The zone of the apical portion (0–0.1 mm) of JE had a higher ratio

Table 1 Effects of toothbrushing stimulation on the density of proliferating cell nuclear antigen (PCNA)-positive cells and total cells

	Density of PCNA-positive cells			*Density of total cells*		
Location	*PR*[a]	*TB*	*P*	*PR*	*TB*	*P*
Basal cells[b]	2.0 ± 0.8[d]	3.5 ± 1.7	0.002	9.3 ± 1.3	10.4 ± 1.0	NS
Fibroblasts[c]						
JE	3.1 ± 1.0	6.2 ± 1.9	0.003	14.5 ± 2.2	16.9 ± 3.1	NS
CM	7.2 ± 3.0	8.4 ± 3.0	NS[e]	27.2 ± 5.4	29.3 ± 5.3	NS
ABP	4.7 ± 2.0	6.2 ± 2.2	NS	22.8 ± 5.0	23.3 ± 4.3	NS
ABO	2.1 ± 0.9	3.7 ± 1.6	0.003	13.4 ± 3.4	14.7 ± 2.7	NS
OE	1.5 ± 0.6	2.5 ± 0.8	0.010	12.9 ± 1.4	13.6 ± 1.8	NS

[a]PR, plaque-removal group; TB, toothbrushing group.
[b]Number of cells per 0.1 mm of basement membrane.
[c]Number of cells per 0.1 mm × 0.1 mm; JE, connective tissue subjacent to the junctional epithelium; CM, connective tissue adjacent to the cementum; ABP, connective tissue adjacent to the alveolar bone of the periodontal ligament side; ABO, connective tissue adjacent to the alveolar bone of the oral epithelial side; OE, connective tissue subjacent to the oral epithelium.
[d]Mean value ± s.d., $n = 12$.
[e]NS, not significant.

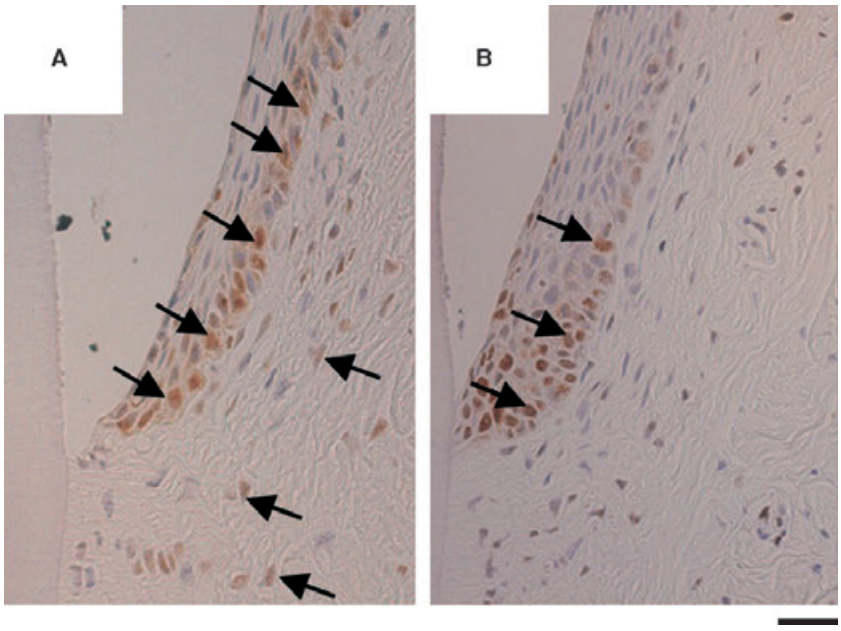

Figure 2 Gingival sulcus area stained for proliferating cell nuclear antigen (PCNA). Except for basal cells in the apical portion of the junctional epithelium, gingiva in the toothbrushing group (A) showed more PCNA-positive cells (arrows, brown-stained nuclei) than the corresponding gingiva in the plaque-removal group (B). The apical zone of the JE had a higher ratio of PCNA-positive basal cells than other zones in the plaque-removal groups (B). Bar, 20 μm

of PCNA-positive basal cells than all other zones, and was the only zone that showed no significant difference between the plaque removal and toothbrushing groups.

In the zone 0 to 1.2 mm towards the coronal side of the CEJ, the PCNA-positive fibroblast ratio was higher in the toothbrushing group than in the plaque-removal group. No significant difference was observed in the apical side of CEJ.

Discussion

Proliferation of basal cells (0.1–1.2 mm from CEJ) in JE was promoted by toothbrushing. The proliferation would reduce gingival bleeding because JE of the periodontal pocket is often ulcerated in periodontitis and the ulcer allows gingival bleeding (Carranza, 2002). These support the clinical findings, which showed the effects of mechanical stimulation by toothbrushing on reduction of gingival bleeding (Fourel *et al*, 1981).

The most apical portion of JE had 60% of PCNA-positive cells and the value was not increased by toothbrushing. The proliferation of the basal cells at the most apical portion of JE plays an important role in apical migration of JE along the root surface. The results suggest that mechanical stimulation by toothbrushing has no effects on apical migration of JE at periodontal pocket formation.

Another explanation for the no change in number of PCNA-positive basal cells of the most apical portion of JE is that the area nearer to CEJ may be already stimulated mechanically without toothbrushing. Because the area is border between free gingiva and attached gingiva, deformation may easily occur when a solid hits the gingiva, for example, at mastication. The stimulation might be responsible to the high number of

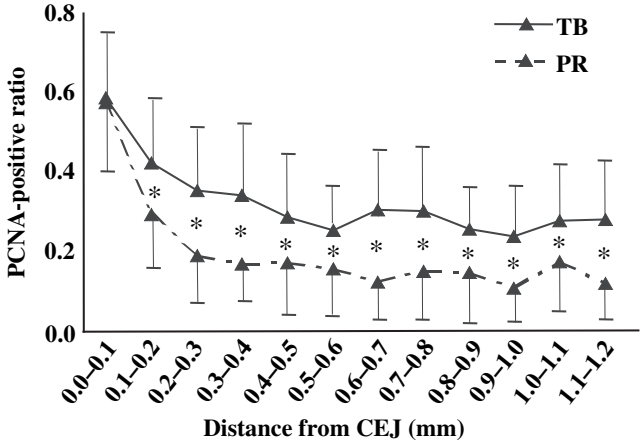

Figure 3 Proliferating cell nuclear antigen (PCNA)-positive basal cell ratios in the junctional epithelium according to the distance from the cemento-enamel junction (CEJ). Mean value ± s.d.; PR, plaque-removal group; TB, toothbrushing group. *Differences were evaluated using Wilcoxon's signed-ranks test ($P < 0.05$)

PCNA-positive basal cells at the most apical portion of JE.

At the initiation of periodontal pocket formation, proliferation of the JE cells close to CEJ along the root surface (Takata and Donath, 1988), reduction in number of gingival fibroblasts (Nemeth *et al*, 1993) and collagen destruction are observed. It is possible that mechanical stimulation by toothbrushing may prevent formation of periodontal pocket, because toothbrushing promoted the proliferative activity of fibroblasts subjacent to JE, but not basal cells of the JE.

The present study shows that toothbrushing promotes the proliferative activity of gingival cells adjacent to the cementum, the alveolar bone of the oral epithelial side and the oral epithelium. However, the proliferative activity of fibroblasts in the periodontium, where alveolar bone prevents the stress, did not change following toothbrushing. The places promoted proliferation of fibroblasts were consistent with stress distribution at toothbrushing (Ito *et al*, 1994). The location of proliferating gingival cells may depend on stress distribution in periodontal tissue. The effects of mechanical stimulation by toothbrushing may not reach to infrabony pocket and furcation involvement.

Alternatively, the difference in promotion of proliferative activity in the different zones of the connective tissues may be due to distinct responses of periodontal ligament fibroblasts and gingival fibroblasts. Gingival fibroblasts may respond to mechanical stimulation more readily than periodontal ligament fibroblasts. In fact, proliferation of gingival fibroblasts is stimulated by platelet-derived growth factor (Dennison *et al*, 1994) and extracellular matrix proteins (Giannopolou and Cimasoni, 1996) *in vitro* to a greater extent than periodontal ligament fibroblasts. In addition, it has been demonstrated that at least three connective tissue progenitor cell populations were present in gingival connective tissue in distinct locations (McCulloch, 1986; Pender *et al*, 1988; McCulloch and Knowles, 1991), and the three kinds of progenitor cells showed different cell cycle time with mechanical stimulation simulating orthodontic tooth movement (Zentner *et al*, 2001). The difference in the composition of the fibroblast subpopulation may account for the variation of cell reaction in different location.

In the connective tissue zones adjacent to the cementum and alveolar bone of the periodontal ligament side, no statistical difference in the number of PCNA-positive fibroblasts was observed between the plaque-removal and toothbrushing groups. These two zones had higher numbers of PCNA-positive fibroblasts than the other zones in connective tissue (Table 1). Also, there may be a limit to the degree to which density of PCNA-positive cells is increased by toothbrushing. These findings are in agreement with those reported previously, where mechanical stimulation by orthodontic force did not affect proliferation of periodontal ligament fibroblasts (Zentner *et al*, 2000).

No change was observed in density of fibroblasts in connective tissue adjacent to cementum and adjacent to the alveolar bone of the periodontal ligament side. These results suggest that mechanical stimulation by toothbrushing has no effects on proliferation of periodontal ligament fibroblasts. Because apoptosis of periodontal ligament fibroblasts are induced by application of periodontal pathogens (Ekuni *et al*,

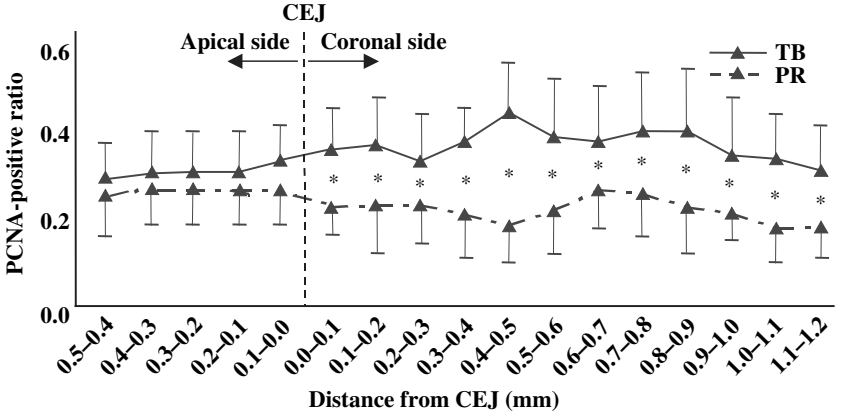

Figure 4 Proliferating cell nuclear antigen (PCNA)-positive fibroblast ratios according to the distance from the cemento-enamel junction (CEJ). Mean value ± s.d.; PR, plaque-removal group; TB, toothbrushing group. *Differences were evaluated using Wilcoxon's signed-ranks test ($P < 0.05$)

2005), toothbrushing alone is not effective to supply new periodontal ligament fibroblasts by promoting proliferation. Removal or reduction of periodontal pathogen is required to restore the periodontal tissues in periodontal ligament.

In conclusion, the mechanical stress of toothbrushing promotes proliferative activity of fibroblasts in the periodontium and gingival cells other than those basal cells in the apical portion of the JE. The repair of periodontal tissue might be promoted by toothbrushing within the limit of the direct mechanical stimulation.

Acknowledgements

The authors are grateful to Prof. Kee-wan Chang for his support. This research was supported in part by the grant of Post-Doctoral Program, Chonbuk National University (2002) and by a grant-in-aid from the Ministry of Education, Science and Culture of Japan (11672045).

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