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Effects of toothbrushing frequency on proliferation of gingival cells and collagen synthesis

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Abstract

Background: Mechanical stimulation by toothbrushing enhances proliferation of fibroblasts and junctional epithelium (JE). These changes in gingiva may depend on the interval between toothbrushing. The effects of toothbrushing frequency on proliferation of gingival fibroblasts and basal cells of JE were evaluated.

Methods: Twelve mongrel dogs were used. Each tooth was brushed for 20 s at 1.96 N. The subepithelial connective tissue of JE was examined for proliferating cell nuclear antigen (PCNA)-positive fibroblasts and procollagen type-I C-peptide (PIP)-positive fibroblasts. JE was examined for PCNA-positive basal cells.

Results: Gingiva that received brushing twice a day showed increases in the density of fibroblasts and ratio of PCNA-positive fibroblasts to total fibroblasts at 4 weeks. The ratio of PIP-positive fibroblasts increased at 8 weeks in gingiva brushed twice a day and once a day. PCNA-positive basal cell ratio increased at 4 weeks in gingiva brushed twice a day and once a day.

Conclusions: A high frequency of brushing was associated with increased numbers of PCNA-positive fibroblasts, PIP-positive fibroblasts and PCNA-positive basal cells. Gingival cell proliferation increased and reached a plateau earlier in gingiva brushed twice a day than in gingiva brushed once a day.

Key words: animal studies; gingivitis; procollagen type I; proliferating cell nuclear antigen; toothbrushing

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Toothbrushing is essential for the prevention and treatment of periodontal disease. Minimum brushing frequency required to establish and maintain periodontal health in a beagle dog model has been evaluated using a gingival index. Brushing three times a week has been found to be sufficient to preserve the health of gingivae (Tromp et al. 1986a). Other studies have found that brushing every day is more effective for establishing and maintaining gingival health than brushing three times a week in dogs with experimental gingivitis (Tromp et al. 1986b) or artificially induced periodontal defects (Corba et al. 1986a, b).

The effects of different frequencies of toothbrushing have also been compared in humans. Stanmeyer's (1957) observational study of 3238 young adults, using a questionnaire and the papilla, marginal and attached gingivae (PMA) index (Schour & Massler 1947), found that inflammation decreases with increasing frequency of brushing, but these benefits appear to peak at a frequency of twice a day. In a 6-week experiment, effective oral hygiene procedures at intervals of 48 h were found to be adequate to maintain the gingival health of students (Lang et al. 1973).

Mechanical stimulation by toothbrushing has been found to promote keratinization of the oral sulcular epithelium (Caffesse et al. 1982), enhance capillary gingival circulation (Brill & Krasse 1959, Hanioka et al. 1993, Perry et al. 1997, Tanaka et al. 1998), cause formation of dense bundles of collagenous connective tissue (Fraleigh 1965) and thicken alveolar bone (Miake et al. 1988). In addition, it has been shown that proliferation of basal cells and fibroblasts and synthesis of procollagen I are promoted more effectively by mechanical stimulation with a toothbrush than by removal of dental plaque in dog gingiva (Horiuchi et al. 2002).

In our previous study, we found that, for cell proliferation in dogs, the most

effective combination of magnitude and duration of toothbrushing once a day is 1.96 N and 20 s (Tomofuji et al. 2002). The frequency of toothbrushing may also play a role in such gingival responses. The present experiment was undertaken to determine the effects of different frequencies of toothbrushing on proliferation of basal cells in the junctional epithelium (JE) and fibroblast proliferation and collagen synthesis in dogs.

Materials and Methods

Experimental design

Twelve mongrel dogs, weighing 8.2–13.4 kg, were used. The mouth of each dog was divided into four quadrants: three for brushing (twice a day, once a day and every 2 days); and one for non-brushed control (plaque removal only). The four brushing frequencies (including non-brushed control) and four durations (0, 4, 8, 12 weeks) were assigned to 16 teeth of each dog by Latin square design (Table 1).

The brushing durations were synchronized so that, for all groups, all brushing or plaque removal was scheduled to cease on the day the dog was killed. For each brushing duration, there was a 2-week pre-experimental period. For example, in the 4-week brushing group, there was an 8-week non-treatment period (neither plaque removal nor brushing), a 2-week pre-experimental period (plaque removal only) and a 4-week experimental period (plaque removal and brushing).

During the 2-week pre-experimental period, supra- and subgingival plaque was removed once a day with a curette (Hu-Friedy Mfg. Co. Inc., IL, USA) from the maxillary second, third and fourth premolars and first molar, and the mandibular third and fourth premolars and first and second molars. The remaining plaque was assessed with disclosing solution and then removed.

During the experimental period, all teeth received supra-/subgingival plaque removal using a scaler twice a day, taking care to touch gingiva as little as possible. The dogs were fed a softened dog-chow DS diet (Oriental Yeast Co., Tokyo, Japan).

Buccal tooth surface and gingiva were brushed after a plastic stent was attached to the tooth to fix the position of the toothbrush and avoid brushing stimulation of adjacent teeth. Brushing force was applied perpendicular to the gingival surface with a reciprocating action of the toothbrush at the marginal and attached gingiva (Tomofuji et al. 2002). The toothbrushing was maintained 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]) (Tanaka et al. 1998). The toothbrush had two rows of nylon bristles, three tufts per row, 50 filaments (diameter = 0.2 mm, length = 11 mm, Tynex 612, Du Pont Filaments, Washington, WV, USA) per tuft (Horiuchi et al. 2002).

The animal experiments were conducted in accordance with institutional guidelines of the Animal Center for Medical Research of Okayama University.

Histological and immunohistochemical analysis

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. Paraffin-embedded bucco-lingual sections (4 μm) were stained immunohistochemically for proliferating cell nuclear antigen (PCNA) or procollagen type-I C-peptide (PIP). Six sections from each tooth were randomly selected for staining: three for PCNA, and three for PIP.

PCNA was stained using a VECTASTAIN Elite ABC kit (Vector Lab., Burlingame, CA, USA) (Takata et al. 1997). The monoclonal antibody against PCNA was diluted 1/200 in phosphate-buffered saline (PBS). The color was developed with 3-3′-diamino benzidine tetrahydrochloride. Sections were counterstained with Mayer's hematoxylin.

The immunohistochemical staining of PIP was performed using the same general procedure as for PCNA, except that the deparaffinized sections were pretreated with 20 mg/l of proteinase K (Sigma Chemical Co., St. Louis, MO, USA). Monoclonal antibody to human PIP (Takara, Kyoto, Japan) was diluted 1/150 in PBS (Horiuchi et al. 2002).

One examiner performed the following histometric analyses using a microscope at 400 × magnification after the preparations were assigned random numbers so that the examiner did not know which treatment each sample had received. PCNA- and PIP-positive fibroblasts were counted in 10 consecutive areas (0.1 mm × 0.1 mm each) adjacent to the basement membrane from the most apical point of the JE (Horiuchi et al. 2002). Ten standard (0.1 mm) areas in the basal cell layer of the JE were defined from the most apical portion to the coronal portion (Tomofuji et al. 2002). The number of

Table 1. Experimental design of the tooth distribution of 12 dogs

Brushing frequency		Group		
		A	B	C
control	once a day	dog no. 1	dog no. 5	dog no. 9
every 2 days	twice a day			
every 2 days	control	dog no. 2	dog no. 6	dog no. 10
twice a day	once a day			
twice a day	every 2 days	dog no. 3	dog no. 7	dog no. 11
once a day	control			
once a day	twice a day	dog no. 4	dog no. 8	dog no. 12
control	every 2 days			

Group A; $\frac{P2 \mid P2}{P3 \mid P3}$ 4 weeks, $\frac{P3 \mid P4}{P4 \mid P4}$ 8 weeks, $\frac{P4 \mid P4}{M1 \mid M1}$ 12 weeks.

Group B; $\frac{P2 \mid P2}{P3 \mid P3}$ 8 weeks, $\frac{P3 \mid P4}{P4 \mid P4}$ 12 weeks, $\frac{P4 \mid P4}{M1 \mid M1}$ 4 weeks.

Group C; $\frac{P2 \mid P2}{P3 \mid P3}$ 12 weeks, $\frac{P3 \mid P4}{P4 \mid P4}$ 4 weeks, $\frac{P4 \mid P4}{M1 \mid M1}$ 8 weeks.

Groups A, B, C; $\frac{M1 \mid M1}{M2 \mid M3}$ 0 weeks.

total and PCNA-positive basal cells was counted in each standard area.

Clinical measurements

The gingival index (Löe & Silness 1963) was measured during the experimental period.

Statistical analysis

All parameters in the 10 standard areas were averaged: numbers of total fibroblasts, PCNA- and PIP-positive fibroblasts, and numbers of total and PCNA-positive basal cells. For each parameter, the mean of three histological sections was calculated for each tooth.

One-way ANOVA and Tukey's method were performed using an SPSS statistical package (10.0 J for Windows, SPSS Japan, Tokyo, Japan).

Results

In both the twice-a-day and once-a-day brushing groups, the density of fibroblasts was significantly greater than that of the control at 4, 8 and 12 weeks (Table 2). There was no statistically significant difference in the density of fibroblasts between the control group and the every-2-days brushing group from 0 to 12 weeks.

At 4 weeks, the ratio of PCNA-positive fibroblasts was higher in the twice-a-day brushing group than in the control group (Table 3). In the every-2-days brushing group, the ratio of PCNA-positive fibroblasts did not significantly exceed that of the control group at the eighth week.

PIP-positive fibroblast ratios are shown in Fig. 1. There were significant differences ($p<0.05$) in the PIP-positive fibroblast ratio between the control group and both the twice-a-day and once-a-day brushing groups at 8 weeks.

The PCNA-positive basal cell ratios of the twice-a-day and once-a-day brushing groups were higher than that of the control group at 4, 8 and 12 weeks (Fig. 2). The epithelial cell density did not change in any of the four groups during the experimental period. The maximum PCNA-positive basal cell ratio of the twice-a-day brushing group occurred at 8 weeks. There was no change in the PCNA-positive basal cell ratio in the every-2-days group or control group.

At the beginning of the experimental period, the mean (with standard deviation) gingival index scores of the four groups were distributed from 0.08 (0.28) to 0.14 (0.42). There were no statistical differences in gingival index scores among the four groups during the experimental period.

Discussion

The ratios of PCNA- and PIP-positive cells of gingiva increased early in gingivae treated with a high frequency of brushing. Although higher frequency of brushing produced earlier responses of fibroblast proliferation and collagen synthesis, there was little difference in the maximum values of these responses. The density of fibroblasts reached a plateau within 8 weeks in the twice-a-day and once-a-day brushing groups. These results suggest that proliferation of fibroblasts and production of collagen type I resulted in acceleration of tissue turnover rather than a net increase in tissue volume; i.e., no gingival hyperplasia (Uzel et al. 2001).

In the every-2-days brushing group, there was no change in fibroblast density, but the PCNA-positive fibroblast ratio increased at 12 weeks. Thus, proliferation of fibroblasts may occur after 12 weeks in gingiva brushed every 2 days. In contrast, the once-a-day and twice-a-day brushing groups had the highest fibroblast density and PCNA-positive fibroblast ratio at 4 weeks, with no significant difference between these two groups. Brushing once a day may be sufficient to promote proliferation of fibroblasts.

An increased ratio of PIP-positive fibroblasts was observed in the once-a-day and twice-a-day brushing groups at 8 weeks. Three weeks of brushing once a day is not sufficient to induce PIP production in dog gingiva (Horiuchi et al. 2002). It may take at least 4 weeks for mechanical stimulation with a toothbrush to induce procollagen synthesis by gingiva, and the reaction reaches a maximum value and plateaus at 8 weeks. However, excretion of functional type-I collagen fibrils was not evaluated in the present study.

In the once-a-day and twice-a-day brushing groups, PCNA-positive basal cell ratios were greater than that of the control group within 4 weeks. Proliferation of epithelial cells promotes repair of ulcerated periodontal pocket epithelium that accompanies gingival bleeding and pus discharge in periodontal disease (Carranza 2002). Although the gingiva of the experimental dogs in this study were clinically healthy, the present findings suggest that brushing twice a day would be effective in promoting healing of an ulcerated epithelium. Brushing once a day might also be effective, but its cell proliferation effects require more time to reach a maximum level and plateau.

There were no differences in clinical scores among the groups during the experimental period. Clinical indices may be less sensitive than histologi-

Table 2. Density of fibroblasts (cells/0.1 mm × 0.1 mm)

Experimental period (weeks)	Control	Brushing frequency		
		every 2 days	once a day	twice a day
0	12.6±1.9*	13.3±1.7	13.3±1.4	12.7±1.6
4	13.2±2.0	13.3±2.2	16.0±2.2**	15.9±1.9**
8	12.9±1.6	14.3±2.1	16.0±2.2**	15.5±0.9**
12	13.8±2.7	14.1±2.2	16.1±2.8**	16.6±2.8**

*Mean ± standard deviation, $n = 12$.
**Significantly greater than the control ($p<0.05$), with Tukey's method.

Table 3. Ratio of PCNA-positive fibroblasts

Experimental period (weeks)	Control	Brushing frequency		
		every 2 days	once a day	twice a day
0	0.213±0.087*	0.207±0.077	0.196±0.071	0.226±0.106
4	0.211±0.117	0.184±0.070	0.315±0.123	0.334±0.085**
8	0.192±0.087	0.248±0.098	0.315±0.088**	0.317±0.074**
12	0.169±0.057	0.276±0.105**	0.274±0.054**	0.310±0.087**

*Mean ± standard deviation, $n = 12$.
**Significantly greater than the control ($p<0.05$), with Tukey's method.

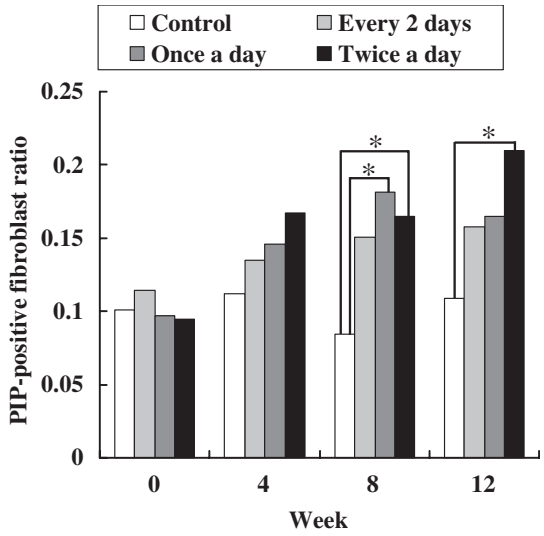

Fig. 1. Ratio of PIP-positive fibroblast to total fibroblasts in the 4 groups. $^{*}p<0.05$, compared to the control group, using Tukey's method.

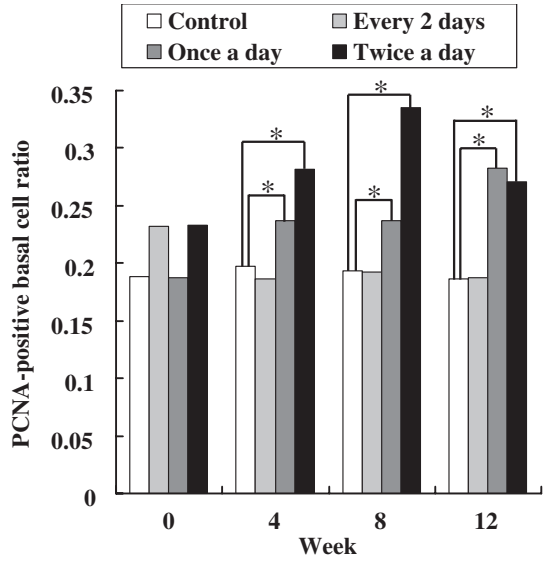

Fig. 2. Ratio of PCNA-positive basal cells of junctional epithelium to total basal cells in the 4 groups. $^{*}p<0.05$, compared to the control group, using Tukey's method.

cal parameters. For example, clinically healthy gingivae have an infiltrate of inflammatory cells, predominantly neutrophils associated with the JE and lymphocytes in the subjacent connective tissue (Page & Schroeder 1976). Even in such a very early stage of inflammation, which is not clinically detectable, collagen depletion within the infiltrated area can be detected. Thus, histological observation provides more precise information about gingival inflammation.

In conclusion, more frequent brushing was associated with increased numbers of PCNA-positive fibroblasts, PIP-positive fibroblasts and PCNA-positive basal cells in JE. These results suggest that brushing at least once a day is necessary to maintain the activity of gingival fibroblasts and JE. Gingival cell proliferation reached a maximum value and plateaued earlier in the twice-a-day brushing group than in the once-a-day brushing group.

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