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Local variations in turnover of periodontal collagen fibers in rats

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Background and Objective: The exact cause of orthodontic relapse is still unclear, although it is often suggested to be caused by periodontal collagen fibers. We hypothesize that long-lived collagen fibers in the periodontium cause relapse. The aim was to determine the half-life of periodontal collagen fibers around rat molars.

Material and Methods: Thirty weanling rats were repeatedly injected with ^{3}H-proline, and autoradiography of histological sections was performed at 1, 4, 8, 15, 22, 29, 36, 57, 78 and 113 d after labeling. Grain densities determined in specific areas of the periodontium were used to calculate collagen half-life.

Results: The half-life ($t_{½}$) was found to decrease from the supra-alveolar region to the apical periodontal ligament region. It was longer in the supra-alveolar region (1.39 ± 0.14 wk) compared with the deeper regions ($p < 0.05$). The $t_{½}$ of the upper periodontal ligament region (0.78 ± 0.20 wk) was longer than that of the inter-radicular periodontal ligament region (0.42 ± 0.07 wk, $p < 0.05$). The $t_{½}$ of the apical periodontal ligament region was 0.61 ± 0.15 wk.

Conclusion: The data indicate that long-lived collagen fibers do not exist in the soft tissues of the periodontium, and are probably not responsible for relapse. The differences in collagen half-life might be caused by local variations in compressive strain induced by normal function.

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One of the main problems in orthodontics is relapse. This is the phenomenon whereby teeth tend to return to their pretreatment position after treatment (1). Ten to 20 years post-treatment, < 30% the patients showed satisfactory alignment (2). In addition, the long-term stability of tooth position is highly variable and unpredictable. Most relapse occurs shortly after removal of the appliance unless retention is applied (3,4). The amount of relapse in the first 3–12 mo post-treatment is indicative of the long-term outcome (5–7).

During relapse, phenomena similar to those during active tooth movement occur. At the former tension side, there is hyalinization of the periodontal ligament (8), osteoclast differentiation and recruitment, and undermining bone resorption (9–12). At the former pressure side, there is osteoblast activity, bone formation (11), and the formation of new periodontal ligament fibers (9). These results suggest that relapse is related to a reversal of the mechanical conditions. Various mechanisms have been proposed, as follows: (i) reorganization of supporting tissues, especially of the fiber system; (ii) muscular imbalance; (iii) unusual tooth morphology and agenesis; and (iv) continued facial growth (1). The first mechanism might be responsible for immediate short-term relapse, while the other factors may be involved in long-term effects (12). Besides these factors, persisting malocclusion shows more relapse due to lack of solid interdigitation, although perfect alignment is no guarantee for stability (1).

The majority of studies examining the short-term effects of relapse focus on the collagen fiber system (13–15); however, elastic and oxytalan fibers have also been studied (16,17). Several decades ago it was suggested that stretched fibers at the former tension side pull back teeth to their pretreatment position (13); however, remodeling of periodontal ligament fibres also occurs during retention (18). It was suggested that relapse is caused by

dentogingival fiber bundles, because they need more time to rearrange than periodontal ligament fibers (4,8,18). Others explained relapse by supra-alveolar collagen fibers that may persist for a longer period of time after experimental tooth movement (13–15). Research in dogs indicates that relapse may also originate from compressed gingival fibers, because they change the mechanical properties of the entire gingival tissue (16,19).

The half-life of collagen fibers around the teeth of rats is described to vary from 1 to 12 d in the periodontal ligament, and from 2 to 152 d for the supra-alveolar fibers (19–23). Although these studies all used a radioactive precursor to determine half-life, the outcome is highly variable, probably due to the use of different techniques, such as autoradiography (23–25) or scintillation counting (20–22,26). We hypothesize that long-lived collagen fibers in specific areas of the periodontium cause relapse after orthodontic treatment. To calculate the half-life of collagen in the periodontium of a rat molar, we determined the rate of disappearance of incorporated ^{3}H-proline. To include all periodontal collagen fibers, repeated labeling started shortly after weaning. The autoradiography method was compared with liquid scintillation counting in skin samples of the same rats.

Material and methods

Animals

Wistar rats were bred at the central animal laboratory of the Radboud University. The animals were housed with one female and her male offspring per cage. A total of 30 weanlings (age 21 d) was used. They were housed in groups of two in normal laboratory conditions, with powdered laboratory rat chow (Sniff, Soest, The Netherlands) and water *ad libitum*. The experiment was approved by the Board for Animal Experiments of the Radboud University Nijmegen, The Netherlands.

Administration of ^{3}H-proline

All weanlings were repeatedly injected intraperitoneally with isotonic saline containing ^{3}H-proline (0.55–1.5 TBq/mmol; Amersham Biosciences, Little Chalfont, UK) at the age of 14, 17, 21, 24, 27, 29, 31, 33 and 35 d. The total amount was 141 μCi (5.2 GBq) ^{3}H-proline per rat, which corresponds to about 0.5 mg of proline. The first injections were given at 14 d of age to ensure labeling of the collagen fibers formed during eruption of the molars. These early interventions had no negative effect on acceptance by the mother, and weight gain of the experimental animals was normal.

Histology and autoradiography

At 1, 4, 8, 15, 22, 29, 36, 57, 78 and 113 d after the last injection of ^{3}H-proline, thus starting from an age of 36 d, groups of three rats were killed by a lethal dose of inhalation anesthesia (isoflurane; Abbott BV, Hoofddorp, The Netherlands) followed by perfusion through the left heart ventricle with fresh 4% paraformaldehyde in 0.1M phosphate-buffered saline, pH 7.4. The maxillae were dissected, and skin biopsies were taken from the back of the animal. The maxillae and half of each skin biopsy were fixed in paraformaldehyde for 24 h at 4°C, then rinsed in phosphate-buffered saline. The other half of each skin biopsy was frozen for liquid scintillation counting.

The maxillae were decalcified in 10% EDTA and embedded in paraffin; the skin samples for autoradiography were treated likewise. Parasagittal 7-μm-thick sections were cut from the molar region of the maxillae, and transverse sections from the skin samples. Every 25th section was mounted on SuperFrost/Plus slides (Menzel-Glaser, Braunschweig, Germany), and stained with hematoxylin and eosin to select suitable sections for autoradiography (Fig. 1A). Selected sections were coated with Kodak NTB-3 emulsion (Kodak Co., Rochester, NY, USA) for thin-layer autoradiography, exposed for 2 mo, and faintly post-stained with hematoxylin and eosin.

Liquid scintillation counting

About 50 mg of back skin from each rat was completely digested overnight at 60°C in 1 mL buffer (pH 6.0) containing 1 mg of papain (Merck, Darmstadt, Germany), 0.2 M NaCl, 0.1 M sodium acetate, 0.01 M L-cysteine HCl and 0.05 M EDTA. Then 300 μL digest was mixed with 3 mL Aqua Luma (Lumac-LSC, Groningen, The Netherlands) and counted in a liquid scintillation counter (LKB-Wallac, Turku, Finland). The total counts per milligram of skin for each time point were expressed as a percentage of the initial counts (= 100%). The half-life was calculated by nonlinear regression analysis (see Statistics section).

Grain count

High-power photomicrographs were taken from 23 predefined areas around the maxillary second molar (Fig. 1B) in selected sections, using a Zeiss Imager Z1 microscope with Zeiss AxioCam MRc5 (Carl Zeiss, Sliedrecht, The Netherlands). For each of the 23 predefined areas, graphs were constructed showing the decrease in grain density over time, using the data from all rats. Grain density in the background of each section was subtracted. One photograph was also made of every skin sample. QWIN software (Leica QWin Pro V2.5; Leica Microsystems Imaging Solutions Ltd, Cambridge, UK) was used to determine the number of grains on the photomicrographs in areas of about 100 μm^2, avoiding areas with blood vessels and artifacts. Data were expressed as number of grains per square micrometer. The 23 areas were clustered into the following four anatomical regions: (A) supra-alveolar (n = 6); (B) upper periodontal ligament (n = 10); (C) inter-radicular periodontal ligament (n = 3); and (D) apical periodontal ligament (n = 4; Fig. 1B).

Statistics

Decay curves were constructed for each of the 23 individual areas (see Fig. 3A) by fitting a nonlinear regression line through the individual data points. The mathematical formula of the regression lines is: $y = y_r + ae^{-bx}$. Where y = grains/area, y_r = grains/

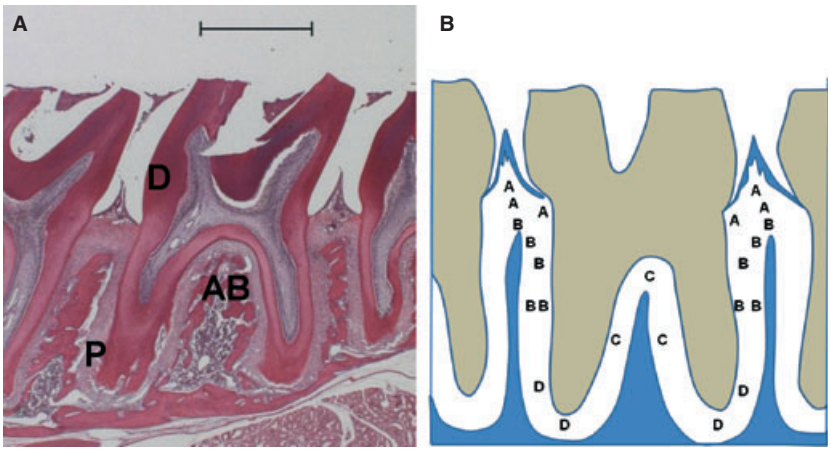

Fig. 1. The rat periodontium. (A) Hematoxylin and eosin staining of a maxillary second molar with its periodontium. Dentin (D), periodontal ligament (P) and alveolar bone (AB) are indicated. The scale bar represents 1000 μm. (B) The different areas of interest are indicated and clustered into a supra-alveolar region (A), an upper periodontal ligament region (B), an inter-radicular periodontal ligament region (C) and an apical periodontal ligament region (D).

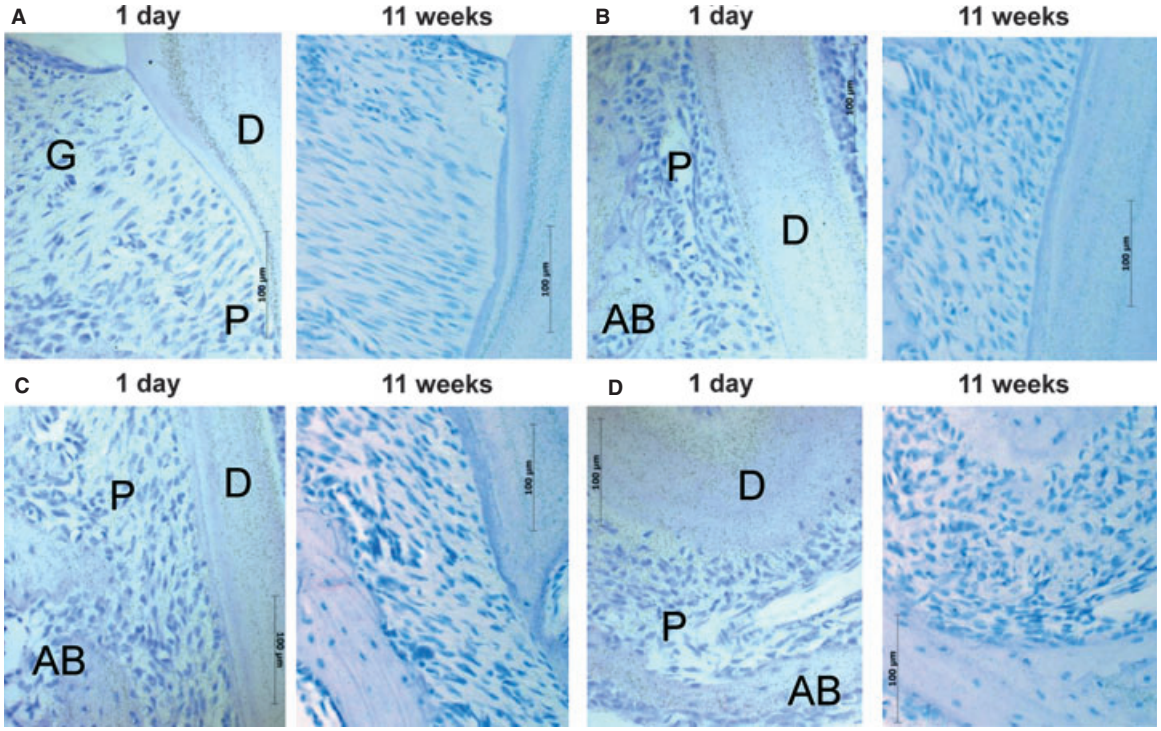

Fig. 2. Autoradiography of anatomical regions. Representative photographs of the four anatomical regions: Supra-alveolar (A), upper periodontal ligament (B), inter-radicular periodontal ligament (C) and apical periodontal ligament (D). The left column shows samples from 1 d after labeling, the right column from 11 wk after labeling. Dentin (D), gingiva (G), periodontal ligament (P), alveolar bone (AB), and cementum (C) are indicated.

area remaining, a = fitting variable, b = fitting variable, e = 2.7183, x = time (weeks). The 95% confidence interval was also plotted for each curve. The half-life ($t_{½}$) for each area was calculated from the corresponding regression line with the mathematical formula: $t_{½} = \ln 2/b$. The individual $t_{½}$ values were then clustered into four anatomical regions, because the confidence intervals of the corresponding regression lines were overlapping. The mean and standard deviation of the $t_{½}$ values was then calculated for each of the four anatomical regions (Fig. 3B).

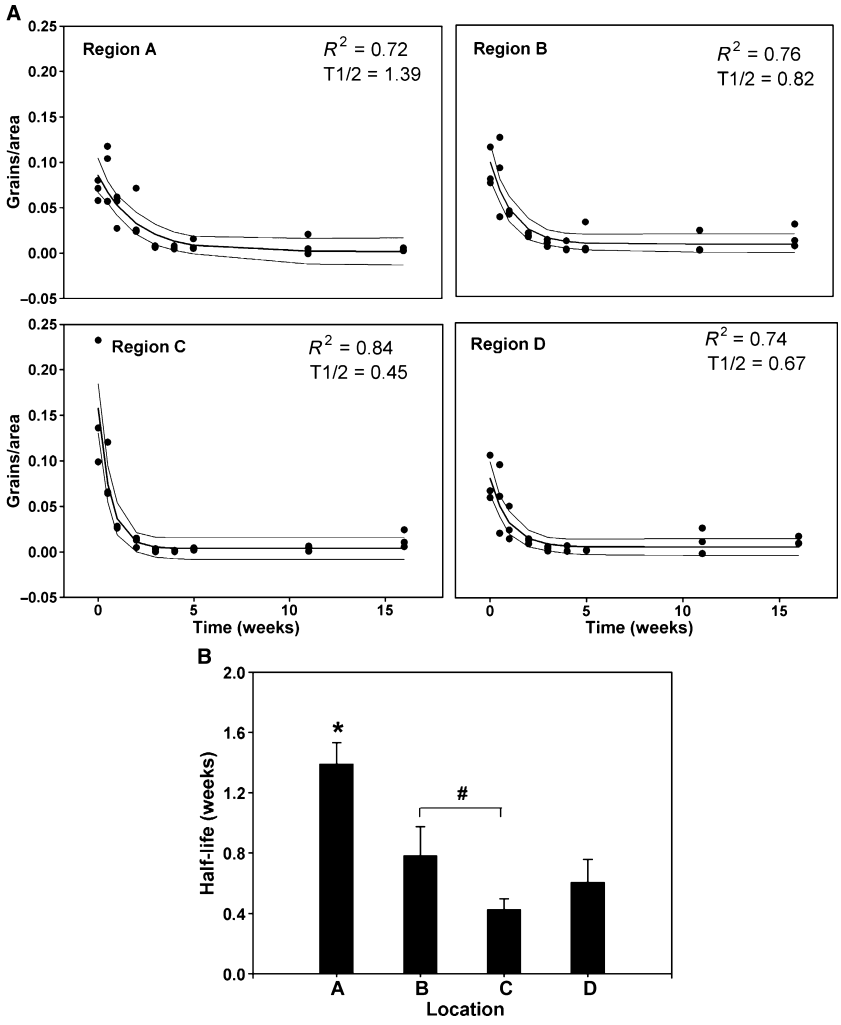

Fig. 3. Half-life of the anatomical regions. (A) Representative decay curves of the following four anatomical regions: supra-alveolar (A), upper periodontal ligament (B), inter-radicular periodontal ligament (C) and apical periodontal ligament (D). The thicker line represents the nonlinear regression line, and the thinner lines the 95% confidence interval. (B) Mean half-life of the four anatomical regions. The half-life in the supra-alveolar region (A) is significantly longer than in the three others regions (*$p < 0.05$). The half-life in the upper periodontal ligament region is significantly longer than in the inter-radicular periodontal ligament region ($p < 0.05$).

Differences between the regions were evaluated by a one-way ANOVA followed by the Holm–Sidak multiple comparisons test, and were considered significant at $p < 0.05$.

Results

Histology

A representative histological section of the upper second molar of a rat is shown in Fig. 1A. The gingiva, periodontal ligament and alveolar bone could be clearly distinguished. In Fig. 1B, the areas of interest are defined as supra-alveolar (A), upper periodontal ligament (B), inter-radicular periodontal ligament (C) and apical periodontal ligament (D) regions.

Autoradiography

Representative autoradiography pictures are shown from the four anatomical regions (A, B, C and D) at 1 d and 11 wk after the end of labeling (Fig. 2). At all time points, a stable band of labeling was observed within the dentin (Fig. 2A, left and right). At 1 d after labeling (lefthand panels), large numbers of grains are visible in all regions. After 11 wk (righthand panels), hardly any grains remained except in the dentin.

Half-life of periodontal collagen

Figure 3A shows representative decay curves from the four anatomical regions. A fast decrease in grain density was observed in the early weeks. The highest initial rate of decrease was found in the inter-radicular periodontal ligament region (C) and the lowest rate in the supra-alveolar region (D). After 5 wk, the grain density in all regions had decreased to about zero.

For each of the 21 areas, the collagen half-life was calculated from the decay curves. Then the mean half-life ($t_{½}$) was calculated for each anatomical region (Fig. 3B). The longest $t_{½}$ (1.39 ± 0.14 wk) was found for the supra-alveolar region, and was significantly higher than the others ($p < 0.05$). The $t_{½}$ in the upper periodontal ligament region (0.78 ± 0.20 wk) was significantly longer than that of the inter-radicular periodontal ligament region (0.42 ± 0.07 wk, $p < 0.05$). Finally, the $t_{½}$ in the apical periodontal ligament region (0.61 ± 0.15) was in between the latter two values, but not significantly different.

Half-life of skin collagen

In skin samples from each rat, the collagen half-life was determined by autoradiography of histological sections as well as liquid scintillation counting of digested skin (Fig. 4). The $t_{½}$ of skin as determined by autoradiography was 1.42 wk (Fig. 4A; R^2 = 0.84), while the $t_{½}$ determined by liquid scintillation counting was 0.99 wk (Fig. 4B; R^2 = 0.89).

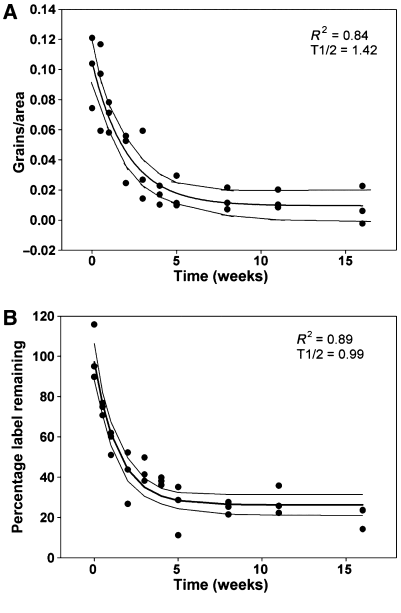

Fig. 4. Collagen half-life in the skin. (A) Half-life is determined by grain counts on autoradiographical sections. (B) Half-life is determined by liquid scintillation counting of digested tissue.

Discussion

The half-life of collagen in different parts of the periodontium of rat molars was determined by autoradiography. We hypothesized that long-lived periodontal collagen fibers might be responsible for orthodontic relapse. The novelty of this study is the definition of specific areas in the periodontium, and the start of labeling prior to eruption of the molars. The latter was done to include possible long-lived fibers that are synthesized in an early stage of periodontal development, which could play a role in relapse. The 23 individual areas were clustered into four anatomical regions. A significantly longer collagen half-life was found for the supra-alveolar region (1.39 wk) compared with the upper periodontal ligament (0.78 wk), inter-radicular periodontal ligament (0.42 wk) and apical periodontal ligament regions (0.61 wk). In addition, the half-life in the upper periodontal ligament region was significantly longer than that in the inter-radicular periodontal ligament region. However, in all areas nearly all label had disappeared after about 5 wk.

In previous studies, collagen half-life has been determined either by autoradiography (23–25) or by liquid scintillation counting (20–22,26). For liquid scintillation counting, periodontal tissue samples have to be pooled, which only gives general data on collagen half-life. We selected autoradiography in our study to determine half-life in specific regions of the periodontium. In addition, we compared the autoradiographical method and liquid scintillation counting in skin samples, in an attempt to validate the former method. Skin is a more homogeneous tissue than the periodontium, and available in much larger amounts. The half-life of skin collagen measured by liquid scintillation counting (0.99 wk) was shorter than that measured by autoradiography (1.42 wk). This might be caused by the inclusion of hair follicles and blood vessels in liquid scintillation counting, while these are excluded in autoradiography. However, this only holds true if the excluded structures have a relatively short collagen half-life. Overall, the half-life of skin collagen was shorter than the 15 d reported by Sodek after liquid scintillation counting (26). That study, however, is based on short-term measurements of incorporation of ^{3}H-proline into collagen, while ours is a long-term autoradiographical study measuring the disappearance of labeled collagen. In addition, Sodek identifies a fast-degrading pool of newly synthesized collagen and a slow-degrading pool of mature collagen, which are combined in our data.

Our data on collagen half-life in the periodontium are in agreement with previous studies using ^{3}H-proline labeling and autoradiography that report a half-life of 2.5–10 d in rats (23–25). The supra-alveolar region showed a longer half-life than the upper periodontal ligament, inter-radicular periodontal ligament and apical periodontal ligament regions. This is consistent with the lower turnover rate of gingival fibers compared with periodontal ligament or transseptal fibers reported earlier (20,22,24,25). The extremely long half-life of 152 d reported for incisor gingiva might be partly explained by the use of a pool-expansion approach, which tends to overestimate half-life of nongrowing gingival tissue (20).

The most interesting finding in our study is that collagen half-life decreases from the supra-alveolar region down to the apical periodontal ligament region. The shortest collagen half-life, however, is found in the inter-radicular periodontal ligament region. These findings might be related to the strain distribution in the periodontal ligament during normal masticatory function. From finite-element and other studies, it appears that compressive strains during vertical loading are highest in the apical periodontal ligament region and in the inter-radicular periodontal ligament region (27,28). In contrast, tensional strains are highest in the cervical areas of the periodontal ligament. Compressive strain might increase collagen turnover in the periodontal ligament, thereby reducing half-life. This is supported by studies showing increased expression of cata-

bolic factors, such as tumor necrosis factor α and MMP1, at the compression side of the periodontal ligament during tooth movement (29,30).

In conclusion, our data indicate that long-lived collagen molecules do not exist in the periodontal soft tissues, thus precluding the putative role of long-lasting collagen fibers in relapse. Alternatively, the elastic fiber system, including the oxytalan fibers, might contain long-lived fibers that contribute to orthodontic relapse (31,32). However, up to now, only limited data are available on a possible mechanical function of these fibers in the periodontal ligament. Further research is therefore required to elucidate the causative mechanism of orthodontic relapse.

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