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Proliferative activities of epithelial and connective tissue cells in the rat periodontal regeneration using argyrophilic nucleolar organizer regions staining

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Background and objective: It is still an open question why long junctional epithelium can proliferate and occupies the root surface following periodontal surgery or experimentally produced periodontitis, and why the epithelium repopulated once on the root surface is replaced by the connective tissue. The aim of this study is to investigate the proliferative activity of the newly formed regenerative connective tissue and long junctional epithelium during wound healing by staining argyrophilic proteins of the nucleolar organizer regions (AgNORs).

Methods: Regenerative connective tissue and long junctional epithelium were experimentally created by insertion of a rubber piece between maxillary molars of rats for 1 week. After removal of the rubber, AgNORs parameters including nuclear area (NA), AgNORs area (AA), AgNORs percentage nuclear area (APNA), AgNORs number (AN) and nuclear number (NN) in regenerative connective tissue and long junctional epithelium were measured and analyzed statistically.

Results: APNA in long junctional epithelium after 1 and 4 weeks was over two times greater than that in the regenerative connective tissue. AA in long junctional epithelium was significantly higher than in regenerative connective tissue at 1 and at 4 weeks post-treatment. AN was higher in the central portion than at the root surface except at 20 weeks. APNA and AA decreased remarkably in long junctional epithelium at 12 weeks post-treatment (approximately half at 4 weeks), whereas in regenerative connective tissue, they did not change distinctly.

Conclusions: These results imply that long junctional epithelium cannot supply sufficient epithelial cells because of their significantly low rates of proliferation, consequently long junctional epithelium becomes shorter after 12 weeks, whereas the proliferative activity of regenerative connective tissue maintains the same level of proliferation, and ultimately long junctional epithelium is replaced by regenerative connective tissue.

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In 1976, Melcher suggested that the cells that repopulate the root surface after periodontal surgery determine the nature of the attachment that will form (1). The curetted root surface may be repopulated by four different types of cells, i.e. epithelial cells, or cells originating from the gingival connective tissue, the bone and the periodontal ligament (2). It is well known that the long junctional epithelium forms clinically on the exposed root surface following periodontal surgery such as root planning and curettage (3–7). Numerous studies have also demonstrated that the long junctional epithelium can be detected in animals with experimentally produced periodontitis (8–18).

It has been reported that once produced on the root surface the long junctional epithelium is replaced with connective tissues during the course of wound healing in two different rat models, created by inserting a rubber piece between the molar teeth or by periodontal surgery (6, 14, 17, 18). Those studies have noted that the rat epithelial attachment with the long junctional epithelium is displaced coronally and that the apical portion of the long junctional epithelium is eventually replaced by connective tissue.

Detailed informations on the proliferative activities of cells relevant to events of regeneration are significant to understand the mechanisms of epithelial and connective tissue attachment. Immunohistochemical stainings for proliferating cell nuclear antigen (19, 20), Ki-67 (21) and argyrophilic nucleolar organizer regions (AgNORs) (18, 22–25) have been utilized to detect proliferating cells. Among these methods, AgNORs staining is believed to be a useful method for examination of nucleolar structure and variations in nucleolar activity. Further, it is regarded to be most suitable for histo-morphometrical analysis using a computer-based imaging process because the grains can be distinguished as variable sized black or brown dots (26). However, few studies have investigated the proliferative activity in the connective tissue during periodontal regeneration employing the AgNORs method.

In a previous study, we used AgNORs staining to examine the epithelium produced experimentally in rats in order to investigate the proliferative activity of the long junctional epithelium (18). It was evident that the AgNORs ratio of the long junctional epithelium was approximately twice of that of the normal junctional epithelium after 4–12 weeks. This suggests that the proliferative activity of the long junctional epithelium is maintained at a continuously high level by epithelial cells, which can migrate directly to the root surface or via the apical portion and finally desquamate from the surface of the epithelium. We have surmised that the long junctional epithelium may decrease in length and be replaced by connective tissue during the turnover of cells with high levels of proliferative activity in the epithelium (18).

Nevertheless, it still opens questions of why the long junctional epithelium can proliferate and occupies most of the root surface following experimentally produced periodontitis or following periodontal surgery, and why the epithelium repopulated once on the root surface is replaced by the connective tissue.

The aim of the present study was to investigate using AgNORs staining the proliferative activity of the cells of connective tissue, which probably originate from the periodontal ligament, compared with that of the long junctional epithelium, which are experimentally created by inserting a rubber piece between maxillary molars of rats. We focused on several parameters of this staining such as nuclear area (NA), AgNORs area (AA), AgNORs percentage nuclear area (APNA) and nuclear number (NN) in the regenerative connective tissue and in the long junctional epithelium. Based on these results, we consider reasons why the long junctional epithelium migrates on the exposed root surface at an early time period and why, once established, the long junctional epithelium is then replaced by the connective tissue from a viewpoint of the proliferative activities of epithelial and connective tissue cells.

Material and methods

Animals and tissue preparation

Twenty-four male Sprague-Dawley rats (9 weeks old), weighing approximately 250 g each, were used in these experiments. Elastic rubber pieces ($1 \times 1 \times 3$ mm) were inserted between the first and second maxillary molars on both sides of each rat in the experimental groups. Control groups were not treated. This study protocol was used in accordance with the animal experiment guidelines of Tokyo Dental College and relevant national laws. The rubbers were removed after 1 week, but no other treatments were performed in tissues where a rubber piece had been inserted. The animals were deeply anesthetized with an intraperitoneal injection of sodium thiopental and killed at intervals of 1, 4, 12, 20 and 28 weeks after the removal of the rubber piece, by transcardially perfusion-fixation with 10% neutral buffered formalin.

AgNORs staining

For AgNORs staining, resected maxillary jaw bones were fixed in the same 10% neutral buffered formalin for 24 h, and were then decalcified in 10% EDTA (ethylendiamine tetraacetic acid-2 Na, Wako Pure Chemical, Osaka, Japan) for 1 week at room temperature. Decalcified tissues were rinsed with 0.05 M phosphate-buffered saline, dehydrated with graded ethanol series, cleared with xylene, and embedded in paraffin blocks. Sections 3 μm thick were cut mesio-distally and were mounted on silane (3-aminopropyl-triethoxysilane, Kanto Chemical Co., Tokyo, Japan) coating slides.

These paraffin-embedded sections were deparaffinized with xylene and then dehydrated with a graded ethanol series. They were rinsed with distilled water and were stained with AgNORs according to the modified method of Smith *et al.* (27–29). The reaction solution, composed of 2% gelatin in 1% aqueous formic acid (Wako Pure Chemical), was mixed in a proportion of 1 : 2 (vol : vol) with 50% aqueous

silver nitrate (Sigma Chemical Co., St Louis, MO, USA). The specimens were incubated with this silver reaction solution at room temperature in the dark room for 30 min. After incubation, they were rinsed with distilled water and were then dipped in 2% chloroauric acid (Wako Pure Chemical) for 10 min for gold toning. The sections were fixed with photograph fixative solution (Fujifix, Fuji Photo Film, Tokyo, Japan) and were rinsed with distilled water. Counterstaining was performed with Mayer's hematoxylin for 30 s. The sections were dehydrated with a graded ethanol series, cleared in xylene and mounted.

Specimens were examined by light microscopy (Axiophot 2, Carl Zeiss, Oberkochen, Germany) and photographed.

Statistical analysis

For histomorphometry, tissue sections were photographed randomly at a final magnification of ×250 by the 35 mm color slide film. The color slides were printed or scanned with a film recorder, and imaging analysis was performed using a personal computer with the public domain NIH Image Program (Version 1.61, developed at the US National Institutes of Health). Data were analyzed statistically using SAS/Stat software (Version 6.03, SAS Institute, Inc.).

Since we examined cells of both long junctional epithelium and fibroblasts, which appeared around the denuded root surface induced by a rubber piece insertion during periodontal regeneration after removal of the rubber piece, we used the term of regenerative connective tissue to the connective tissues (Fig. 1). The estimated areas in the untreated specimens were basal cells of the normal junctional epithelium and cells of the normal periodontal ligament. The latter was measured in the 50 μm square located just adjacent to the root surface (root surface connective tissue) and adjoining the 50 μm central square (central connective tissue) (Fig. 1A). The measured areas in the experimental specimens were basal cells of the long junctional epithelium and regenerative connective tissue. The latter was estimated in the 50 μm square of both at the root surface (root surface regenerative connective tissue) and central (central regenerative connective tissue). These areas were exclusively located at a distance of 50 μm from the apical ends of the epithelium (Fig. 1B).

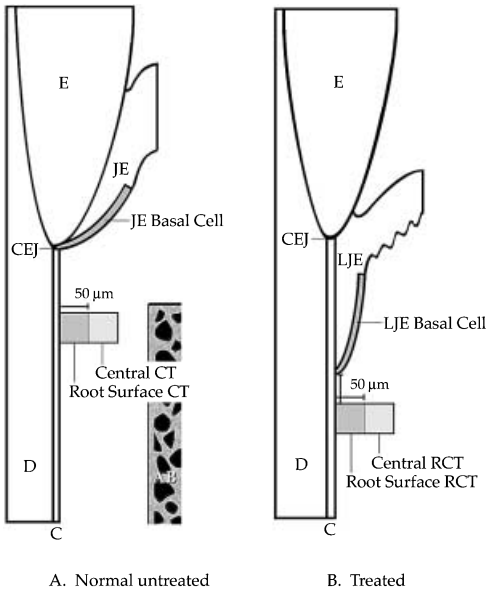

Fig. 1. Schema indicating the measurement area of junctional epithelium, long junctional epithelium, connective tissue and regenerative connnective tissue. In untreated specimens, the argyrophilic proteins of the nucleolar organizer regions (AgNORs) percentage nuclear area (APNA), AgNORs area (AA) and AgNORs number (AN) were measured in basal cells of the normal junctional epithelium and cells of the normal periodontal ligament (A). Those parameters were also examined in basal cells of the long junctional epithelium and regenerative connective tissue cells from 1 to 28 weeks post-treatment in the experimental groups. Both the AN and the NN were estimated in the 50 μm^2 areas located just adjacent to the root surface (root surface regenerative connective tissue) and adjoining the 50 μm^2 central areas (central regenerative connective tissue). The estimated areas were exclusively situated at a distance of 50 μm from the apical ends of the epithelium (B). AB, alveolar bone; C, cementum; CEJ, cemento-enamel junction; CT, connective tissue; D, dentin; E, enamel; JE, junctional epithelium; LJE, long junctional epithelium; RCT, regenerative connective tissue.

(i) The NA signifies the average area per nucleus and was calculated as follows:

$$\mathrm{NA}(\mu m^2) = \Sigma(\text{nuclear area})/\text{number of nuclei.} \quad (1)$$

(ii) The AA indicates the total area occupied by AgNORs granules in the nucleus and was calculated according to the following equation.

$$\mathrm{AA}(\mu m^2) = \Sigma(\text{AgNORs area})/\text{number of nuclei.} \quad (2)$$

Knowing both the NA and the AA enabled the APNA to be calculated.

(iii) The APNA means the proportion of the nuclear area occupied by AgNORs and was calculated by the following equation.

$$\mathrm{APNA}(\%) = \text{AgNORs area}/\text{nuclear area} \times 100. \quad (3)$$

(iv) The AgNORs number (AN) is the mean number of AgNORs per nucleus and was calculated as follows:

$$\mathrm{AN}(n) = \Sigma(\text{AgNORs number})/\text{number of nuclei.} \quad (4)$$

(v) NN(n) was counted the number of nuclei in the 50 μm square areas and indicated as values per 100 μm^2.

Besides, we eliminated the nuclei that could not be estimated by the AA in the image analysis process in the measurement of APNA and AA,

because both APNA and AA are required to calculate the AgNORs area.

We examined (i) each area in every group compared with the control group and (ii) each area in every group compared with each other. In untreated specimens, we measured the APNA, AA and AN in basal cells of the normal junctional epithelium and cells of the normal periodontal ligament. We calculated also those parameters in basal cells of the long junctional epithelium and regenerative connective tissue cells from 1 to 28 weeks post-treatment in the experimental groups (Fig. 1). Both the AN and the NN were estimated in the 50 μm^2 areas located just adjacent to the root surface (root surface regenerative connective tissue) and adjoining 50 μm^2 central areas (central regenerative connective tissue). The estimated areas were exclusively situated at a distance of 50 μm from the apical ends of the epithelium (Fig. 1). Regenerative fibroblasts were observed in the submucosal connective tissue area after 1 week.

Data for each group were compared using Tukey's studentized range (honestly significant difference) test ($p < 0.05$). Total estimated numbers of basal cell nucleus of the long junctional epithelium were 44 in untreated, 100 at 1 week, 169 at 4 weeks, 60 at 12 weeks, 61 at 20 weeks, 46 at 28 weeks, and fibroblast nucleus of regenerative connective tissue were 192 in untreated, 312 at 1 week, 160 at 4 weeks, 149 at 12 weeks, 134 at 20 weeks, 198 at 28 weeks.

Results

AgNORs observation in both regenerative connective tissue cell and basal layer of the epithelial cell nucleus

One week after the rubber removal, regenerative oral epithelium had proliferated to cover the surface of granulation tissue. The epithelial attachment with the long junctional epithelium was established in 4 weeks (Fig. 2A) and was displaced coronally and consequently long junctional epithelium became shorter at 12–28 post weeks (Fig. 2B). After displacement of the long junctional epithelium, root surface was covered by connective tissue attachment associated with newly formed cementum (data is not shown).

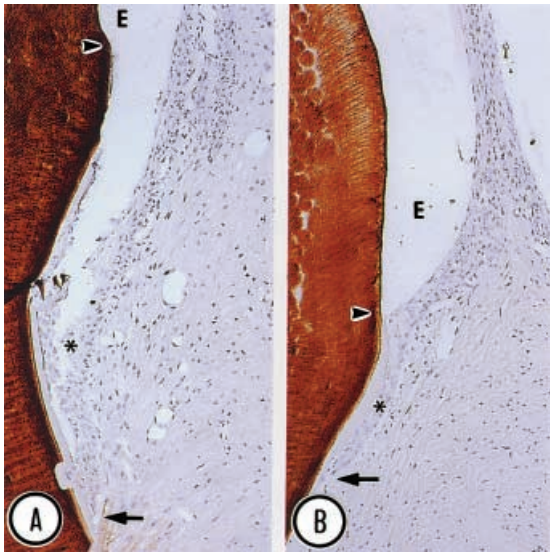

Fig. 2. Low magnification micrographs of long junctional epithelium. The epithelial attachment with the long junctional epithelium was established at 4 weeks post-treatment (A). On the 28 weeks post-treatment, the long junctional epithelium became obviously shorter in length (B). Asterisks indicate long junctional epithelium, arrows show the apical end of long junctional epithelium and arrow heads display cemento-enamel junction. E, enamel space (argyrophilic nucleolar organizer regions staining, original magnification ×125).

In the control group, AgNORs were recognized as small black particles or grains with variable size and number in the nuclei of junctional epithelium cells and of connective tissue cells. This staining was exclusively specific and background precipitation was slight and negligible. One to three small and smooth AgNORs particles (round in shape) were discernible in each connective tissue cell nucleus (Fig. 3A). Particles of the cell nucleus located close to alveolar bone tissue were larger than those at the root surface side (Fig. 3B). On the contrary, one or two relatively large sized dots were observed in the basal layer of the untreated junctional epithelium (Fig. 4A).

One week after the removal of the rubber piece, regenerative connective tissue, which should be termed as granulation tissue with severe inflammation, was evident on the exposed root surface of both the first and second maxillary molars. The tissue, which had abundant cellular elements, was composed of immature fibroblast-like cells, dilated blood vessels and inflammatory cells. The surface of the regenerative connective tissue was covered by regenerative oral epithelium. AgNORs particles in the regenerative connective tissue were remarkably increased in number and size compared with those in untreated periodontal ligament cells (Fig. 3C). Extremely large and irregular shaped AgNORs particles were found in regenerative epithelium 1 week after the removal of the rubber piece (Fig. 4B).

At 4 weeks post-removal, the long junctional epithelium was established on the exposed root surface, and connective tissue attachment was definite at the root surface adjacent to the apical portion of the long junctional epithelium. The number and size of AgNORs particles in the regenerative connective tissue cells were almost analogous to those in the tissue after 1 week (Fig. 3D). In the long junctional epithelium cells, however, the size and number of the particles became smaller than those in the epithelium after 1 week, particularly at the interface of root surface (Fig. 4C).

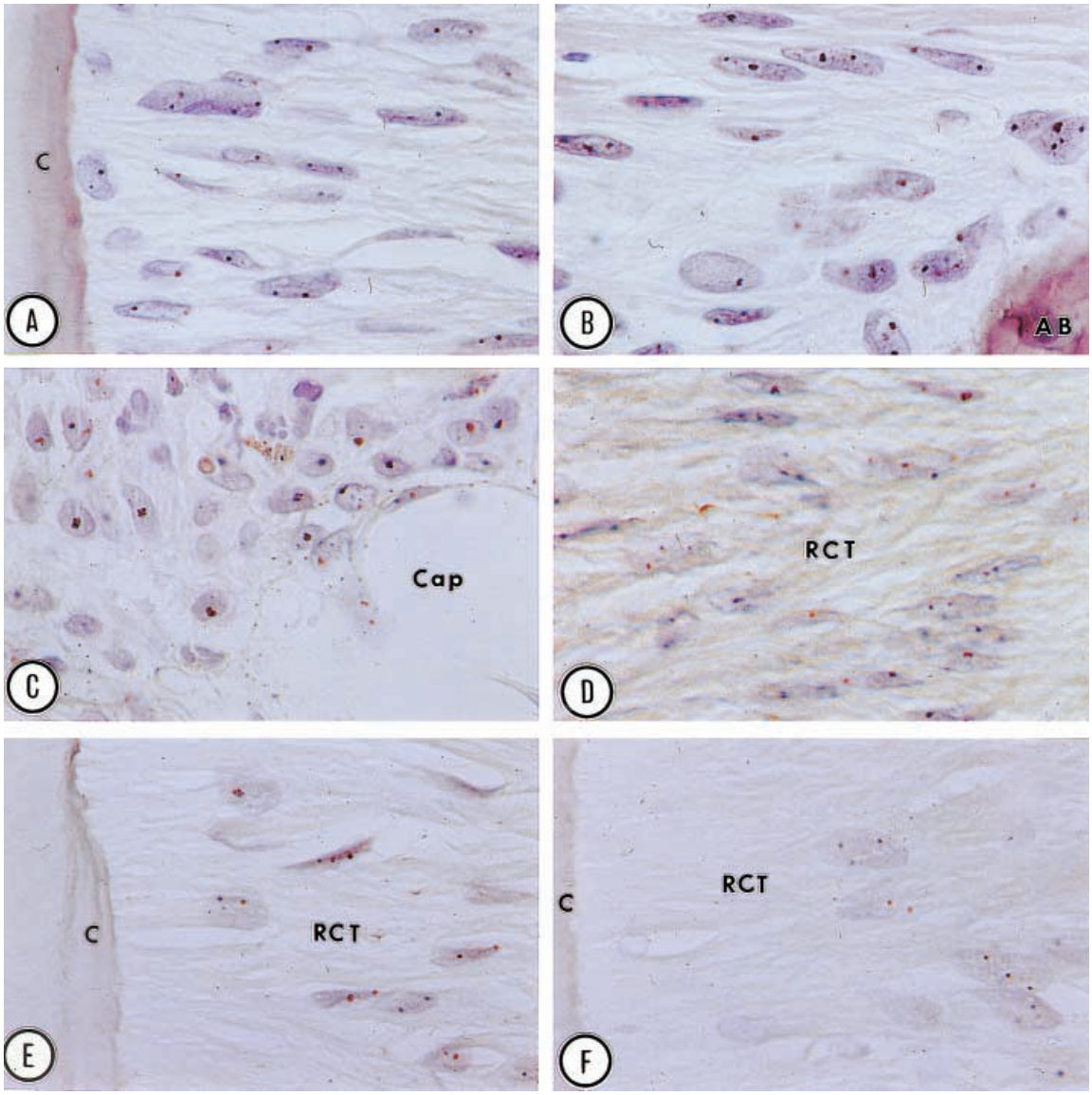

Fig. 3. Micrographs of argyrophilic proteins of the nucleolar organizer regions (AgNORs) in untreated periodontal ligament and regenerative connective tissue. AgNORs were recognized as small black particles or grains with variable size and number on the nucleus of untreated periodontal ligament and regenerative connective tissue. Small particles are distinct in nuclei of cells of the normal periodontal ligament at the root surface side (A) and central side (B). The number and size of AgNORs particles were increased remarkably in the granulation tissue after 1 week (C) and in regenerative connective tissue after 4 weeks (D). After 12 weeks, a distinct connective tissue attachment associated with newly formed cementum and thick collagen fibers was discernible. Both number and size of AgNORs particles were decreased in the cell nucleus of the connective tissue attachment (E). The size and number of AgNORs showed a tendency to decrease after 28 weeks (F). AB, alveolar bone; C, cementum; Cap, capillary; RCT, regenerative connective tissue (original magnification ×800).

In contrast, the cell density of these regenerative connective tissues was significantly decreased.

After 12 weeks, a distinct connective tissue attachment associated with the newly formed cementum and thick collagen fibers (resembling Sharpey's fibers) was discernible by the shortening and movement of the long junctional epithelium to the coronal side. Both the number and the size of AgNORs particles were decreased in the cell nucleus of the connective tissue attachment (Fig. 3E). The apical ends of the long junctional epithelium, which had been established on the exposed root surface, had moved progressively to the coronal side, and the long junctional epithelium became smaller in length and width than those of the epithelium after 4 weeks. The size and number of the particles were also lesser in the long junctional epithelium after 12 weeks than those in the epithelium after 4 weeks (Fig. 4D).

At both 20 and 28 weeks post-treatment, the long junctional epithelium became obviously shorter in length and the denuded root surface was covered over time by newly produced cementum associated with thick collagen fibers resembling Sharpey's fibers. The size and number of

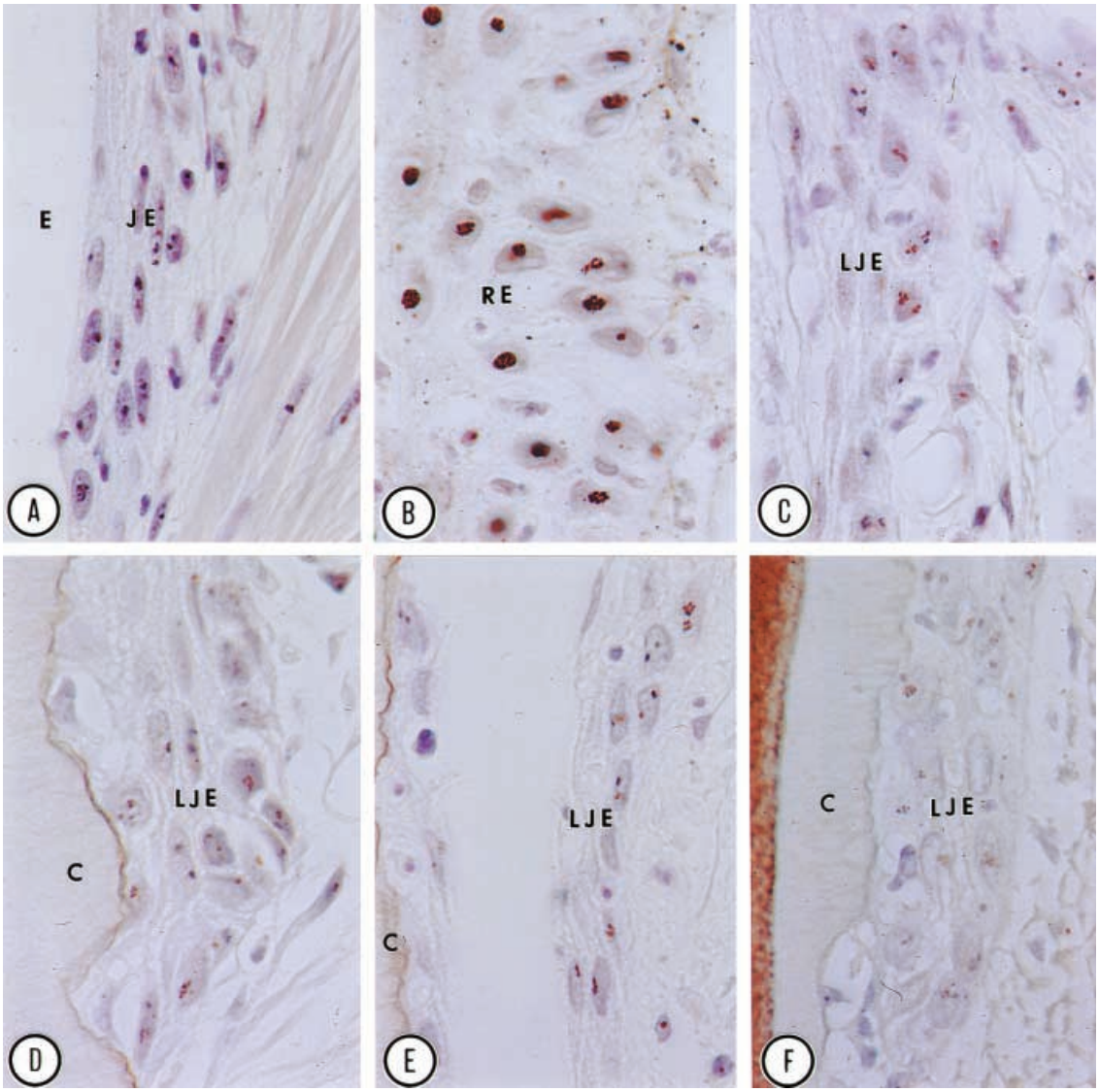

Fig. 4. Micrographs of argyrophilic proteins of the nucleolar organizer regions (AgNORs) in untreated junctional epithelium and long junctional epithelium. Relatively large sized one or two AgNORs particles were observed in the basal layer of the untreated junctional epithelium (A). Extremely large and irregular shaped AgNORs particles were found in regenerative epithelium at 1 week post-treatment (B). In the established long junctional epithelium cells at 4 weeks, the size and number became smaller, particularly at the interface of the root surface (C). The long junctional epithelium became smaller in length and width, and AgNORs size and number were also lesser in the long junctional epithelium after 12 weeks (D). The size and number of AgNORs particles showed a tendency to decrease and they became smaller and fewer at 20 weeks (E) and 28 weeks post-treatment (F). C, cementum; E, enamel space; JE, junctional epithelium; LJE, long junctional epithelium; RE, regenerative epithelium (original magnification ×800).

AgNORs particles showed a tendency to decrease and they became smaller and fewer (Fig. 3F). Cellular elements in the connective tissue attachment were also decreased. However, relatively larger size and higher quantity of AgNORs particles were detected at the connective tissue interfaces in the long junctional epithelium as well as in the untreated junctional epithelium (Figs 4E and F).

Statistical analysis of AgNORs and nucleus

Statistical analysis of APNA in the regenerative connective tissue cells and long junctional epithelium basal cells — We calculated the mean proportion of nuclear area occupied by AgNORs in regenerative connective tissue cells and basal cells of the long junctional epithelium from 1 to 28 weeks post-treatment, although the regenerative connective tissue cells and basal cells at 1 week post-treatment should be regarded as granulation tissue and regenerative epithelium during healing processes.

The APNA, of cells in periodontal ligament was 4.27 ± 0.10 ($n = 67/125$) and was 6.83 ± 1.20 ($n = 44/70$) in basal cells of junctional epithelium

in the untreated specimens. APNA in regenerative connective tissue was 5.90 ± 1.86 ($n = 141/265$) and that in long junctional epithelium was 12.73 ± 1.23 ($n = 81/123$) after 1 week. Values for APNA in regenerative connective tissue and long junctional epithelium were 5.96 ± 1.27 ($n = 92/187$) and 12.44 ± 1.43 ($n = 111/217$) at 4 weeks post-treatment, 5.27 ± 1.20 ($n = 62/132$) and 6.74 ± 1.16 ($n = 49/86$) at 12 weeks, 3.79 ± 0.97 ($n = 53/77$) and 5.51 ± 0.39 ($n = 47/68$) at 20 weeks, 3.49 ± 1.60 ($n = 58/92$) and 5.60 ± 1.72 ($n = 46/87$) at 28 weeks, respectively (n = total estimated number of nuclei/AgNORs).

In the regenerative connective tissue group, the APNA revealed relatively high values in 1, 4 and 12 weeks; however, there was no statistically significant difference among any of the experimental periods. The APNA was the highest in the basal cells of the epithelium after 1 week and then in the newly formed long junctional epithelium after 4 weeks. These values were nearly two times higher than that in the untreated junctional epithelium. In contrast, from 12 to 28 weeks, the APNA at each experimental period was almost identical to the control group. Significant differences were statistically evident in the control vs. 1 week and 4 weeks, and at 4 weeks vs. 12, 20, and 28 weeks (Fig. 5, $p < 0.05$).

Comparing the connective tissue with the junctional epithelium, the APNA in the junctional epithelium was approximately 1.6 times higher than the connective tissue in the untreated control groups. APNA in long junctional epithelium after 1 and 4 weeks was over two times greater than that in the regenerative connective tissue. However, those ratios became similar to the untreated tissues with the passage of time after 12 weeks in both regenerative connective tissue and the long junctional epithelium.

Statistical analysis of mean AA in regenerative connective tissue cells and long junctional epithelium basal cells — The total areas occupied by AgNORs granules in regenerative connective tissue cells and in basal cells of the long junctional epithelium from 1 to 28 weeks post-treatment were calculated. However, the regenerative connective tissue cells and basal cells after 1 week corresponded to the granulation tissue and the regenerative epithelium in the course of regeneration.

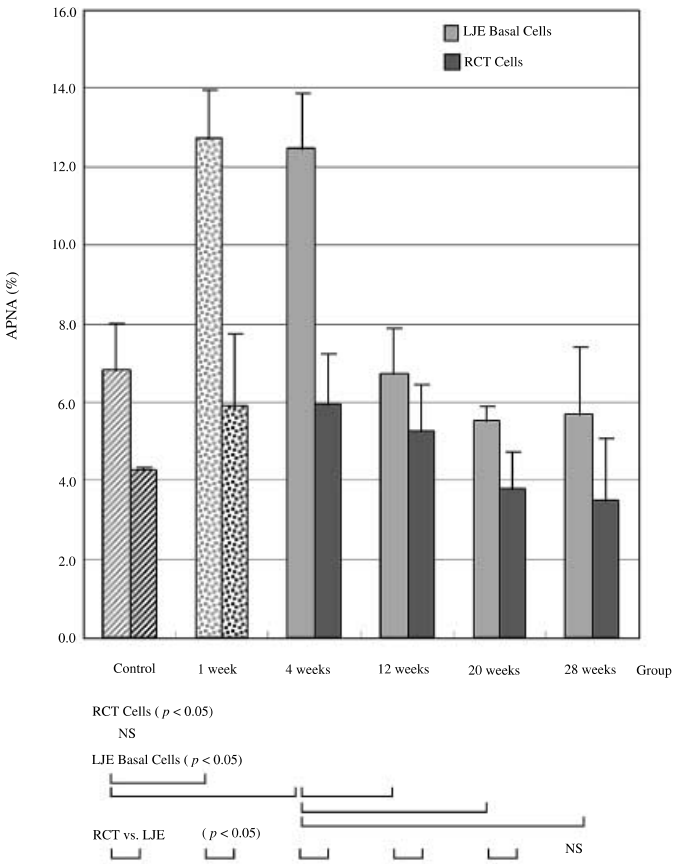

Fig. 5. Statistical analysis of argyrophilic proteins of the nucleolar organizer regions percentage nuclear area (APNA) in the regenerative connective tissue (RCT) cells and long junctional epithelium (LJE) basal cells. Values are mean ± SD.

The AA (μm^2) of cells in periodontal ligament was 1.17 ± 0.18 ($n = 67/125$) and 1.24 ± 0.15 ($n = 44/70$) in basal cells of junctional epithelium in the untreated specimens. AA in regenerative connective tissue was 1.50 ± 0.46 ($n = 141/265$) and that in regenerative oral epithelium was 4.48 ± 0.45 ($n = 81/123$) after 1 week. Values for AA in regenerative connective tissue and long junctional epithelium were 1.47 ± 0.23 ($n = 92/187$) and 3.09 ± 0.39 ($n = 111/217$) at 4 weeks post-treatment, 1.32 ± 0.29 ($n = 62/132$) and 1.27 ± 0.29 ($n = 49/86$) at 12 weeks, 0.94 ± 0.14 ($n = 53/77$) and 1.00 ± 0.33 ($n = 47/68$) at 20 weeks, 0.72 ± 0.19 ($n = 58/92$) and 1.17 ± 0.23 ($n = 46/87$) at 28 weeks, respectively (n = total estimated number of nuclei/AgNORs).

In the regenerative connective tissue cells group, values of AA were slightly higher at 1 and at 4 weeks, although the difference was not statistically significant between the control and 1–4 weeks. At 4 weeks, the area had decreased with time, and significant differences were discernible between 4 weeks and 20–28 weeks ($p < 0.05$).

The AA was high in basal cells of the regenerative epithelium after 1 and after 4 weeks. The AA at 1 week was approximately 3.6 times that in the untreated junctional epithelium, and the value at 4 weeks was approximately 2.5 times higher. On the other hand, the AA values from 12 to 28 weeks were almost the same as the control group. Differences were statistically significant between the control vs. 1 and 4 weeks, and between 4 weeks vs. 12, 20 and 28 weeks (Fig. 6, $p < 0.05$).

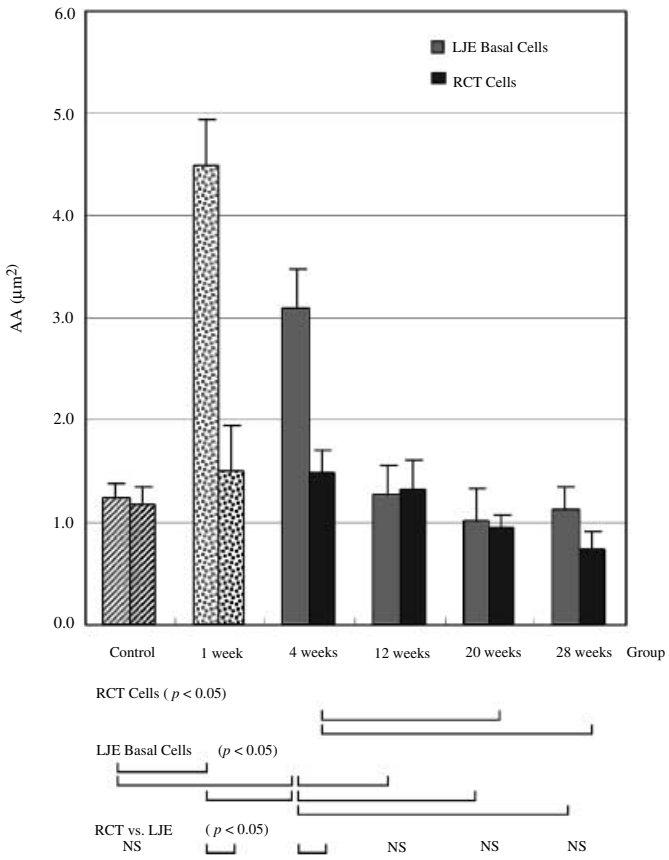

Fig. 6. Statistical analysis of mean of argyrophilic proteins of the nucleolar organizer regions area (AA) in the regenerative connective tissue (RCT) cells and long junctional epithelium (LJE) basal cells. Values are mean ± SD.

When the AA in the regenerative connective tissue was compared with that in long junctional epithelium, the value in the connective tissue was almost identical to the junctional epithelium in control groups. The AA in the regenerative connective tissue was slightly higher at 1, 4 and 12 weeks post-treatment, whereas AA in the long junctional epithelium was markedly higher after 1 and 4 weeks. The AA in the long junctional epithelium was remarkably higher than in the regenerative connective tissue at 1 week and 4 weeks post-treatment.

Statistical analysis of mean AN in regenerative connective tissue cells and long junctional epithelium basal cells — We calculated the mean number of AgNORs particles per nucleus in the regenerative connective tissue cells and in basal cells of the long junctional epithelium from 1 to 28 weeks post-treatment. However, regenerative connective tissue cells and basal cells at 1 week post-treatment might be regarded as granulation tissue and regenerative epithelium that appeared during the healing processes.

The AN of cells of junctional epithelium in the untreated specimens was 1.63 ± 0.23 ($n = 44/70$) and that of regenerative oral epithelium was 1.61 ± 0.38 ($n = 100/161$) after 1 week. Values for AN in long junctional epithelium were 1.73 ± 0.33 ($n = 169/294$) at 4 weeks, 1.64 ± 0.40 ($n = 60/102$) at 12 weeks, 1.58 ± 0.38 ($n = 61/95$) at 20 weeks, and 1.89 ± 0.08 ($n = 46/87$) at 28 weeks. The AN of cells of periodontal ligament in the control animals was 2.12 ± 0.83 ($n = 192/407$) and those of regenerative connective tissue were 2.68 ± 1.28 ($n = 312/839$) at 1 week, 2.68 ± 0.96 ($n = 160/428$) at 4 weeks, 2.10 ± 0.98 ($n = 149/313$) at 12 weeks, 1.60 ± 0.64 ($n = 134/214$) at 20 weeks, and 1.47 ± 0.62 ($n = 198/291$) at 28 weeks, respectively, (n = total estimated number of nuclei/AgNORs).

Only two AgNORs particles in each nucleus were counted in the untreated periodontal ligament cells. In the regenerative connective tissue cells, the AN values were the highest at 1 week and at 4 weeks. They were approximately 1.3 times the number in untreated periodontal ligament and the differences were statistically significant between the control and 1–4 weeks (Figs 7, $p < 0.05$). After 12 weeks, the AN decreased slightly and was similar to the untreated periodontal ligament. The AN values decreased over time at 20 weeks and 28 weeks, and were statistically lower than the untreated periodontal ligament (Fig. 7, $p < 0.05$).

The AN in the basal cells showed no significant difference between the newly formed long junctional epithelium at any experimental period and the untreated junctional epithelium. There were significant differences in the number of long junctional epithelium between 4 weeks vs. 12, 20 and 28 weeks, and between 12 weeks vs. 20 weeks (Fig. 7, $p < 0.05$).

The AN values in the regenerative connective tissue were slightly higher than in the long junctional epithelium at 1–12 weeks post-treatment and in the untreated groups.

Statistical analysis of the AN of the regenerative connective tissue cells in the areas of both root surface and center in regenerative connective tissue — The mean AN values of regenerative connective tissue cells in areas of the root surface and the center in the periodontal ligament were calculated in each experimental period. However, the regenerative connective tissue cells after 1 week correspond to granulation tissue, and subsequently it was difficult to classify the tissue into the root surface and the central portion.

The AN values in root surface and central area of periodontal ligament were 1.94 ± 0.84 ($n = 90/175$) and 2.27 ± 0.79 ($n = 102/232$) in the untreated animals. AN values in these areas were 2.44 ± 0.81 ($n = 64/156$)

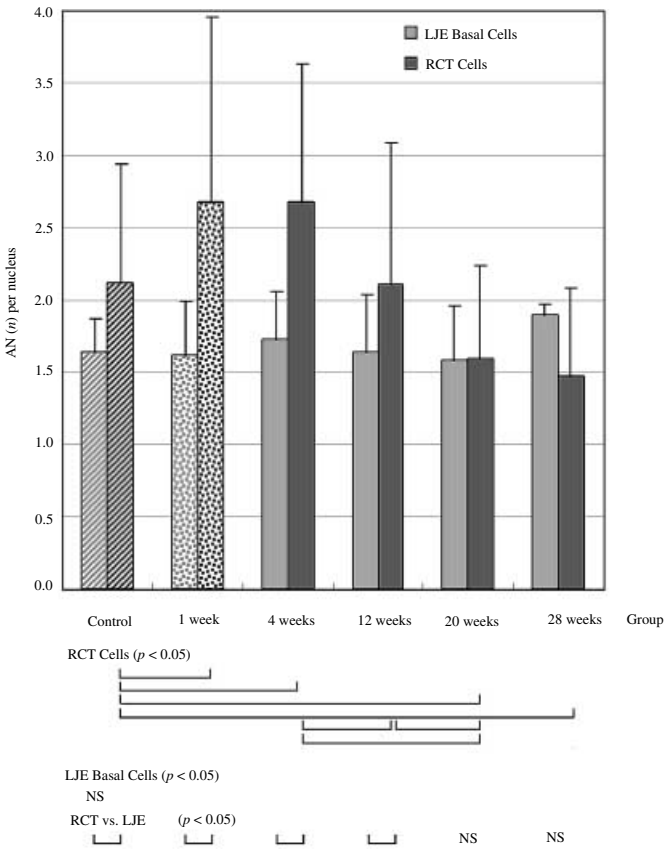

Fig. 7. Statistical analysis of mean numbers of argyrophilic proteins of the nucleolar organizer regions per nucleus (AN) in the regenerative connective tissue (RCT) cells and long junctional epithelium (LJE) basal cells. Values are mean ± SD.

and 2.83 ± 1.01 ($n = 96/272$) at 4 weeks post-treatment, 1.93 ± 0.90 ($n = 55/106$) and 2.20 ± 1.02 ($n = 94/207$) at 12 weeks, 1.64 ± 0.66 ($n = 42/69$) and 1.58 ± 0.63 ($n = 92/145$) at 20 weeks, and 1.37 ± 0.53 ($n = 97/133$) and 1.56 ± 0.68 ($n = 101/158$) at 28 weeks, respectively. (n = total estimated number of nuclei/AgNORs).

The AN was higher in the central portion than at the root surface in the control and experimental periods except for 20 weeks, although none of the differences were statistically significant between the central portion and the root surface.

At the root surface, there were statistically significant differences between the control vs. 4 weeks and 28 weeks, and also between the 4 weeks vs. 12, 20 and 28 weeks (Fig. 8, $p < 0.05$). At the central regenerative connective tissue, statistically significant differences were seen not only between the control vs. 4, 20 and 28 weeks, but also between 4 weeks vs. 12, 20 and 28 weeks, and between 12 weeks vs. 20 weeks (Fig. 8, $p < 0.05$).

Statistical analysis of mean NN of the regenerative connective tissue in the areas of both root surface and the central regenerative connective tissue — We statistically analyzed the NN of regenerative connective tissue cells at the root surface, and in the central regenerative connective tissue and total regenerative connective tissue in each experimental period. However, regenerative connective tissue cells after 1 week should be regarded as granulation tissue, therefore we estimated only the total regenerative connective tissue area because it was difficult to classified the tissue into the root surface and the central portions.

The values for NN in root surface, central and total area of periodontal ligament were 50.97 ± 4.92; 46.34 ± 7.74; 48.46 ± 6.12 in the control. The NN of the total area was 131.82 ± 4.15 at 1 week post-treatment. Values for NN in the root surface, central and total areas of regenerative connective tissue were 53.31 ± 5.05, 55.79 ± 9.21, 54.94 ± 5.79 at 4 weeks, 36.83 ± 6.63, 44.67 ± 2.55, 41.31 ± 3.32 at 12 weeks, 26.71 ± 5.85, 36.06 ± 4.65, 32.00 ± 4.67 at 20 weeks, 26.54 ± 6.19, 25.83 ± 7.11, 25.92 ± 5.10 at 28 weeks, respectively.

The NN in total regenerative connective tissue (indicating regenerative connective tissue cell density) was the highest in the granulation tissue that appeared at 1 week post-treatment. It was approximately 2.7 times that of the control group, and this was a statistically significant difference (Fig. 9, $p < 0.05$). The NN rapidly decreased after 4 weeks, and thereafter became similar to the control.

After 12 weeks, the NN decreased over time and those of the total regenerative connective tissue became lower than those of the untreated periodontal ligament as well as at 20 and 28 weeks ($p < 0.05$). The NN values of the root surface revealed statistically low levels at 12, 20 and 28 weeks post-treatment, and that of the central area after 28 weeks, compared with the control group ($p < 0.05$). Those levels in total regenerative connective tissue corresponded to 0.85 times, 0.66 times and 0.53 times of 12, 20 and 28 weeks post-treatment, respectively.

The NN of regenerative connective tissue at the root surface side was lower than in the central regenerative connective tissue after 12 and 20 weeks, and this was statistically significant ($p < 0.05$).

Discussion

Regenerative connective tissue and long junctional epithelium

In a previous study, we observed the regenerative events that follow the insertion of a rubber piece between the molars of rats for 1 week (18). Severe inflammation and ulceration associated with bone resorption and root surface exposure was caused between

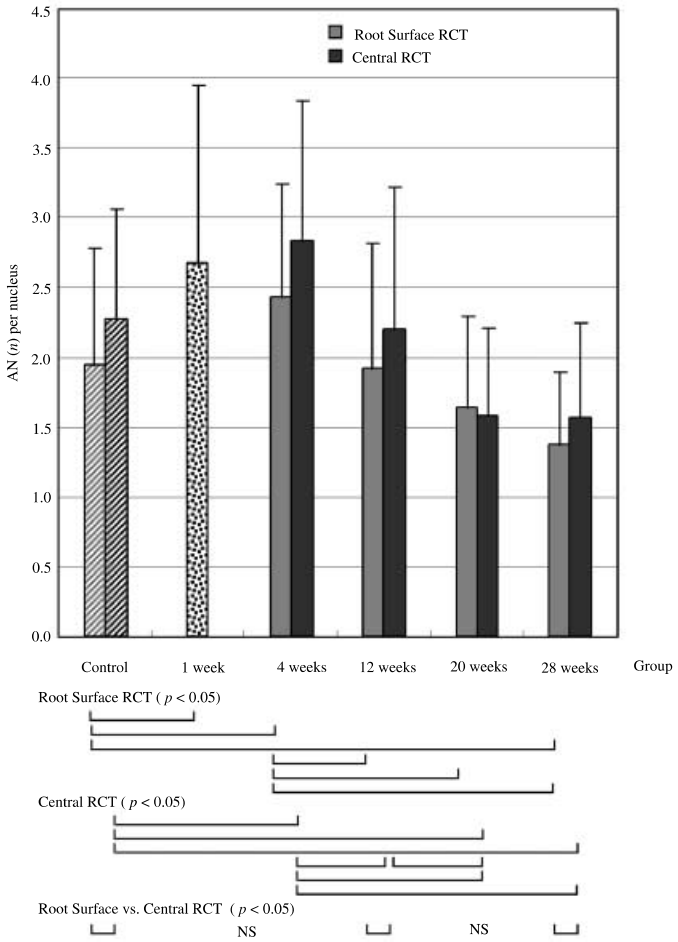

Fig. 8. Statistical analysis of mean numbers of argyrophilic proteins of the nucleolar organizer regions per nucleus (AN) of the regenerative connective tissue (RCT) cells in the areas of both root surface and central RCT. Values are mean ± SD.

the first and second maxillary molars. The regenerative epithelium had migrated to cover the granulation tissue 1 week after the removal of the rubber, and a distinct long junctional epithelium could be detected on the exposed root surface at 4 weeks post-treatment. The apical ends of the long junctional epithelium had moved to the coronal side and connective tissue attachment associate with newly formed cementum was found on the root surface at 12 weeks post-treatment. The connective tissue attachment increased in relative volume with the passage of time, and ultimately the long junctional epithelium became shorter at 24 and 28 weeks.

Our results imply that the epithelial attachment by the long junctional epithelium, which is frequently formed on the root surface at the early stage of the regeneration following periodontal surgery, can be replaced by the connective tissue attachment at the late stage. Therefore, it seems to be unnecessary to obtain the connective tissue attachment immediately after the treatment, and clinical treatments to interrupt the epithelial migration using some kinds of membranes may not be significant for establishing the connective tissue attachment. Nevertheless, we should take into account the great difference between an experimental model of rats employed in this study and cases of human periodontitis.

We consider that gingival connective tissues completely disappeared due to inflammation induced by the rubber insertion in this study, as seen in the previous study. It is surmised, therefore, that the regenerative connective tissue, which appeared in the destroyed tissue during tissue healing, may have originated from the pre-existing periodontal ligament. It has been confirmed that the periodontal ligament is essential for regeneration of periodontal tissues including osteogenesis and cementogenesis (2, 30).

Significance of parameters of AgNORs

AgNORs, which are portions of DNA encoding ribosomal RNA, are located in the nucleoli of all cells and are transcribed by RNA polymerase I, ultimately directing protein synthesis (31, 32). Transcriptionally active nucleolar organizer regions (NORs) associate with certain specific, acidic non-histone proteins that are involved in RNA synthesis, are selectively stained (23, 33) by a silver colloid technique, and can then be visualized as black dots (AgNORs) using an optical microscope (34). Therefore, it is believed that silver staining marks transcriptionally active NORs (35–37).

To quantitatively assess proliferative activity utilizing AgNORs staining, various parameters, including the NA, the AN, the AA and the APNA, have been examined (18, 21, 38, 39). It has been reported that the AN reflects the degree of cellular proliferative activity (18, 31, 40) and differentiation (41). Studies have also demonstrated that the APNA most accurately reflects the proliferative status of a cell (21, 38). Schwint *et al.* have reported that AA and total AA indicate transcriptional activation of previously inactive NOR (39). Other investigators have suggested that AA correlates closely with the proliferative activity (18, 41).

Based on these previous studies, we selected and examined the following parameters, i.e. NA, AA, APNA, AN and NN. To compare the proliferative activity of regenerative connective tissue with that of long junctional epithelium, we investigated chiefly NA, AA, and APNA. AN and NN were examined to analyze the activity at the root surface area and at the central portion in the regenerative connective tissue, because cells of connective tissue contained rel-

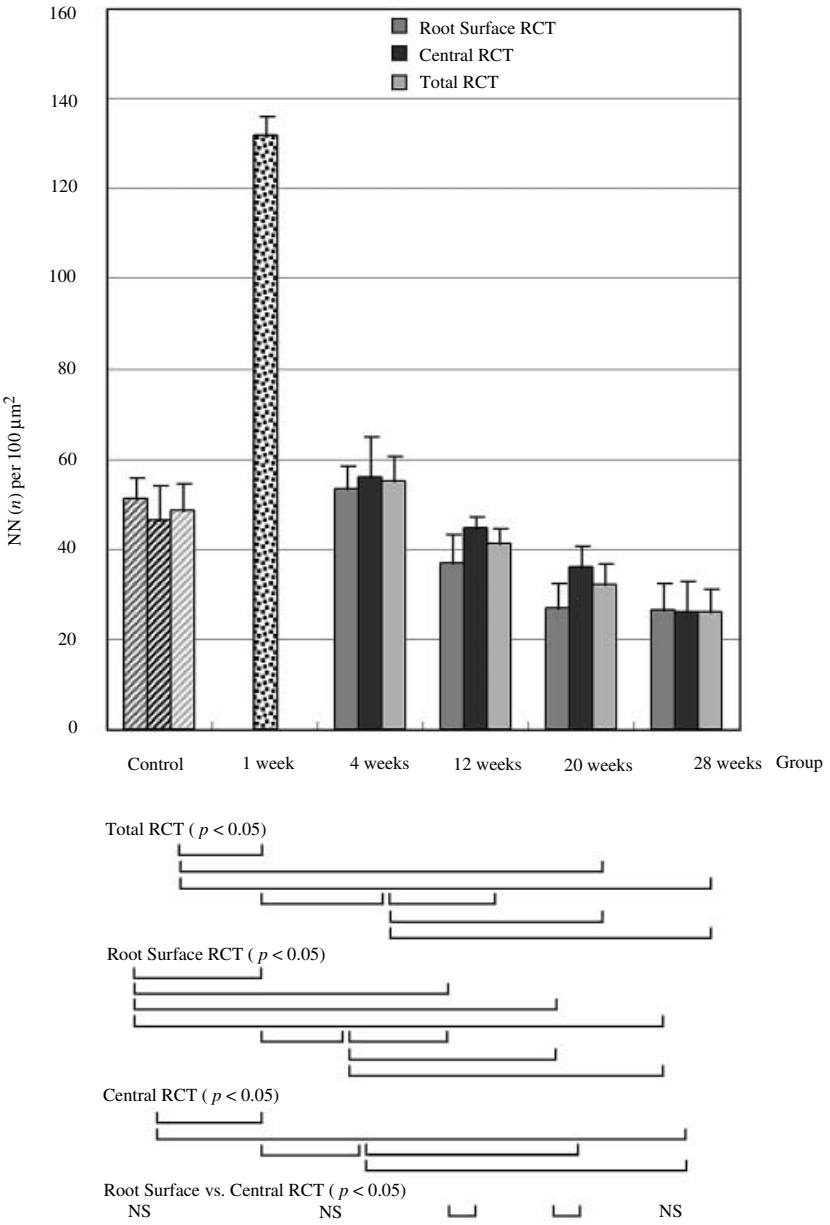

Fig. 9. Statistical analysis of mean nucleus numbers (NN) of the regenerative connective tissue (RCT) in the areas of both root surface and central RCT. Values are mean ± SD.

atively large number of particles, but not large area. Therefore, we considered it is appropriate to use AN and NN, which estimated the number, rather than AN and APNA, which calculated AgNORs area.

To compare the proliferative activity between the regenerative connective tissue and the long junctional epithelium, we initially estimated the parameters between the untreated periodontal ligament and the junctional epithelium, which might indicate the normal proliferation activity of those tissues. Consequently, both the AA and the AN were almost identical in the periodontal ligament and in the junctional epithelium (AA: 1.17 ± 0.18 μm^2 in the periodontal ligament, 1.24 ± 0.15 μm^2 in the long junctional epithelium; AN: 2.12 ± 0.83 in the periodontal ligament, 1.63 ± 0.23 in the long junctional epithelium), although the APNA in the junctional epithelium (6.83 ± 1.20%) was significantly higher than that in the periodontal ligament (4.27 ± 0.10%). These results imply that both the periodontal ligament and the junctional epithelium possess similar proliferative activities under normal physiological conditions.

Taking the regenerative changes in the treated portions of the periodontal tissues at 1 and 4 weeks post-treatment into account, it is easy to understand how the proliferative activity of the regenerative connective tissue and the long junctional epithelium interact during the repair of those tissues. Among the parameters measured in this study, the AA and the APNA reflected remarkably the chronological regeneration events, although the AN indicated only slight changes. The APNA results agree with investigations by Weeks *et al.* (38) and Munakata and Hendricks (21) who have demonstrated that the APNA most accurately reflects the proliferative status of a cell. Our results on the AA are also supported by a study of squamous epithelium which showed that a marked rise in total AA indicates transcriptional activation of previously inactive NOR (39).

APNA and AA in regenerative connective tissue and long junctional epithelium

Since both the APNA and the AA in long junctional epithelium were higher than in the regenerative connective tissue at 1 and 4 weeks post-treatment, we surmise that the long junctional epithelium at the early stage of the regeneration possesses remarkably high proliferative activity in supplying epithelial cells, so that subsequently the long junctional epithelium was able to become established on the exposed root surface. In contrast, the proliferative activity reflected by the APNA and the AA in the regenerative connective tissue was not so high, although they were raised slightly. We suggest that these differences in proliferative activity cause the formation of the long junctional epithelium on the exposed root surface following periodontal surgery (3–7) and in experimentally created periodontitis (8–18).

It is noteworthy that the APNA and the AA decrease remarkably in the long junctional epithelium at 12 weeks post-treatment (approximately half at 4 weeks), whereas those in the regenerative connective tissue do not change and maintain their levels at 4 weeks post-treatment. These may imply that: (i) the long junctional epithelium cannot supply sufficient numbers of epithelial cells because of their low proliferative activity, and consequently the long junctional epithelium becomes shorter at 12 weeks post-treatment; (ii) the proliferative activity of the regenerative connective tissue is not changed and maintains the same level of activity as at 4 weeks post-treatment; (iii) thus, ultimately the long junctional epithelium is replaced by the regenerative connective tissue.

AN and NN in both regenerative connective tissue and untreated periodontal ligament

We examined the AN and the NN, which indicates cell density, in areas of the root surface and in the center of the periodontal ligament. The ANs were higher in the central portion than at the root surface in the control and in the experimental periods except at 20 weeks, although none of the differences were statistically significant between the central portion and the root surface. The NN in the total regenerative connective tissue was the highest in the granulation tissue which appeared at 1 week post-treatment. It was approximately 2.7 times higher than the control group, and statistically significant differences were evident (Fig. 6, $p < 0.05$). The NN rapidly decreased after 4 weeks, and became quite similar to the control. However, the NN in the area of the root surface was higher than in the center of the periodontal ligament in the untreated control group. These results are consistent with a study by Inoue *et al*. (42), who demonstrated a significantly higher number of cells near the cementum in the untreated periodontal ligament of beagle dogs. We suggest that the cell density is the highest at the root surface area and is the lowest at the central area of periodontal ligament under normal physiological conditions, whereas the proliferation activity is the highest at the central area. These may involve the turnover of periodontal ligament cells whose proliferating cells migrate to the cementum surface (43). Our results on the AN and the NN in the regenerative connective tissue and in the untreated periodontal ligament may support the idea of such a turnover of the periodontal ligament.

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References

1. Melcher AH. On the repair potential of periodontal tissues. *J Periodontol* 1976;**47:**256–260.
2. Nyman S, Lindhe J, Karring T. Reattachment–new attachment. In: Lindhe J, eds. *Textbook of Clinical Periodontology*. Copenhagen: Munksgaard, 1983: 410–432.
3. Caton JG, Zander H. The attachment between tooth and gingival tissues after periodic root planning and soft tissue curettage. *J Periodontol* 1979;**50:**462–466.
4. Caton J, Nyman S. Histometric evaluation of periodontal surgery. (I). The modified Widman flap procedure. *J Clin Periodontol* 1980;**7:**212–223.
5. Caton J, Nyman S, Zander H. Histometric evaluation of periodontal surgery. (II). Connective tissue attachment levels after four regenerative procedures. *J Clin Periodontol* 1980;**7:**224–231.
6. Listgarten MA, Rosenberg S, Lerner S. Progressive replacement of epithelial attachment by a connective tissue junction after experimental periodontal surgery in rats. *J Periodontol* 1982;**53:**659–670.
7. Nyman S, Lindhe J, Karring T, Rylander H. New attachment following surgical treatment of human periodontal disease. *J Clin Periodontol* 1982;**9:**290–296.
8. Waldo CM. Method for the study of tissue response to tooth movement. *J Dent Res* 1953;**32:**690–691.
9. Waldo CM, Rothblatt JM. Histologic response to tooth movement in the laboratory rat – procedure and preliminary observation. *J Dent Res* 1954;**33:**481–486.
10. Hunter N, Schwab JH, Simpson DM. Experimental periodontitis induced in rats by streptococcal cell wall fragments. *J Periodont Res* 1979;**14:**453–466.
11. Deporter DA, Brown DY. Fine structural observations on the mechanism of loss of attachment during experimental periodontal disease in the rat. *J Periodont Res* 1980;**16:**304–313.
12. Garnick JJ, Singh B, McKinney RV. Maintenance of long junctional epithelium in the rat. *J Dent Res* 1982;**61:**681–685.
13. Sanavi F, Listgarten MA, Boyd F, Sally K, Nowotny A. The colonization and establishment of invading bacteria in periodontium of ligature-treated immunosuppressed rats. *J Periodontol* 1985;**56:**273–280.
14. Abiko Y, Shimono M. Regeneration of periodontal tissue following experimentally induced periodontitis in rats. *Bull Tokyo Dent Coll* 1989;**30:**195–204.
15. Abiko Y, Shimono M. An ultrastructural study of the pocket epithelium in rats. *Bull Tokyo Dent Coll* 1991;**32:**27–34.
16. Sashima M, Satoh M, Suzuki A. Age-related development of the long-junctional epithelium in the sedescence-accelerated mouse. *J Dent Res* 1991;**70:**1462–1466.
17. Katayanagi T. Histopathological study of wound healing following experimentally induced periodontal pocket in rats. Interaction between epithelial and connective tissue attachments. *Shikwa Gakuho* 1992;**92:**1627–1650 [in Japanese].
18. Uno T, Hashimoto S, Shimono M. A study of the proliferative activity of the long junctional epithelium using argyrophilic nucleolar organizer region (AgNORs) staining. *J Periodont Res* 1998;**33:**298–309.
19. Garcia RL, Coltrera MD, Gown AM. Analysis of proliferative grade using anti-PCNA/cyclin monoclonal antibodies in fixed, embedded tissues. Comparison with flow cytometric analysis. *Am J Pathol* 1989;**134:**733–739.
20. Mabuchi R, Matsuzaka K, Shimono M. Cell proliferation and cell death in periodontal ligaments during orthodontic tooth movement. *J Periodont Res* 2002;**37:**118–124.
21. Munakata S, Hendricks JB. A multilabeling technique for simultaneous demonstration and quantitation of Ki-67 and nucleolar organizer regions (AgNORs) in paraffin-embedded tissue. *J Histochem Cytochem* 1994;**42:**789–793.
22. Polton D, Bobichon H, Adnet JJ. Ultrastructural localization of NOR in nucleoli of human breast cancer tissues using a one-step Ag-NOR staining method. *Biol Cell* 1982;**43:**229–232.
23. Polton D, Menager M, Jeannesson P, Himber G, Pigeon F, Adnet JJ. Improvement in the staining and in the visualiza-

tion of the argyrophilic proteins of the nucleolar organizer region at the optical level. *Histochem J* 1986;**18:**5–14.
24. Crocker J, Skilbeck N. Nucleolar organizer region associated proteins in cutaneous melanotic lesions: a quantitative study. *J Clin Pathol* 1987;**40:**885–889.
25. Abe Y. A histomorphometric study of AgNORs in cellular alterations of junctional and pocket epithelia in experimental periodontitis. *Jpn J Oral Biol* 1996;**38:** 21–31.
26. Kawase N, Shiokawa A, Ota H, Saitoh T, Yoshida H, Kazama K. Nucleolar organizer regions and PCNA expression in prostatic cancers. *Pathol Int* 1994;**44:**213–222.
27. Smith R, Crocker J. Evaluation of nucleolar organizer region associated proteins in breast malignancy. *Histopathology* 1988;**12:**113–125.
28. Sano K, Takahashi H, Fujita S *et al*. Prognostic implication of silver-binding nucleolar organizer regions (AgNORs) in oral squamous cell carcinoma. *J Oral Pathol Med* 1991;**20:**53–56.
29. Abiko Y, Muramatsu T, Tanaka Y *et al*. Basaloid-squamous cell carcinoma of the oral mucosa: report of two cases and study of the proliferative activity. *Pathol Int* 1998;**48:**460–466.
30. Shimono M, Ishikawa T, Ishikawa H *et al*. Regulatory mechanisms of periodontal regeneration. *Microsc Res Tech* 2003;**60:**491–502.
31. Crocker J, Nar P. Nucleolar organizer regions in lymphomas. *J Pathol* 1987; **151:**111–118.
32. Leong AS, Gilham P. Silver staining of nucleolar organizer regions in malignant melanoma and melanotic nevi. *Hum Pathol* 1989;**20:**257–262.
33. Courvalin JC, Hernandez-Verdun D, Gosti-Testu F, Marty MC, Maunoury R, Bornens M. A protein of Mr 80,000 is associated with the nucleolus organizer of human cell lines. *Chromosoma* 1986;**94:** 353–361.
34. Howat AJ, Giri DD, Cotton DW, Slater DN. Nucleolar organizer regions in Spitz nevi and malignant melanomas. *Cancer* 1989;**63:**474–478.
35. Ochs RL, Busch H. Further evidence that phosphoprotein C23 (110 kD/pI 5.1) is the nucleolar silver staining protein. *Exp Cell Res* 1984;**152:**260–265.
36. de Capoa A, Baldini A, Marlekaj P *et al*. Hormone-modulated rRNA gene activity is visualized by selective staining of the NOs. *Cell Biol Int Rep* 1985;**9:**791–796.
37. Wachtler F, Hopman AH, Wiegant J, Schwarzacher HG. On the position of nucleolus organizer regions (NORs) in interphase nuclei. Studies with a new, non-autoradiographic in situ hybridization method. *Exp Cell Res* 1986;**167:**227–240.
38. Weeks SC, Beroukas D, Jarvis LR, Whitehead R. Video image analysis of AgNOR distribution in the normal and adenomatous colorectum. *J Pathol* 1992; **166:**139–145.
39. Schwint AE, Gomez E, Itoiz ME, Cabrini RL. Nucleolar organizer regions as markers of incipient cellular alterations in squamous epithelium. *J Dent Res* 1993;**72:**1233–1236.
40. Jan-Mohamed RM, Armstrong SJ, Crocker J, Leyland MJ, Hulten MA. The relationship between number of interphase nors and nor-bearing chromosomes in non-Hodgkin's lymphoma. *J Pathol* 1989; **158:**3–7.
41. Cabrini RL, Schwint AE, Mendez A, Femopase F, Lanfranchi H, Itoiz ME. Morphometric study of nucleolar organizer regions in human oral normal mucosa, papilloma and squamous cell carcinoma. *J Oral Pathol Med* 1992; **21:**275–279.
42. Inoue T, Hashimoto S, Usuda J, Shimono M. A study on homeostatic factors in periodontal ligament of the beagle dog. Immunohistochemical study of cell and vascular distribution and density. *Jpn J Oral Biol* 1993;**35:**485–495.
43. McCulloch CA, Melcher AH. Continuous labeling of the periodontal ligament of mice. *J Periodont Res* 1983;**18:**231–241.