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The behavior of stem cells and progenitor cells in the periodontal ligament during wound healing as observed using immunohistochemical methods

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Background and Objective: The aim of this study was to identify stem cells or progenitor cells in the periodontal ligament and to investigate their behavior during wound healing of bone defects created experimentally in the alveolar process.

Material and Methods: Intradentinal cavities were created in the mesial root of the first molar of 25 adult male rats that were killed 1, 3, 5, 7 and 14 d after surgery. At each time-point, sections were stained immunohistochemically for CD44s (standard), CD34, c-KIT, PCNA, Cbfa-1 and 5-bromo-2-deoxyuridine using primary antibodies. For morphometric analysis, the ratios of Cbfa-1 and PCNA-positive cells were calculated from the total number of positive cells/10^4 μm^2 in the cavities.

Results: 5-Bromo-2-deoxyuridine-positive cells were observed in the periodontal ligament and had migrated into the wound areas. A small number of CD44s-, CD34- and c-KIT-positive cells were observed in the bone marrow, but none were observed in the periodontal ligament. CD44s-positive cells were only observed in the alveolar bone cavity at 5 d after surgery. CD34- and c-KIT-positive cells were only observed in the dentin cavity at 7 d after surgery. Cbfa-1 and PCNA scores tended to show an increase 7 d after surgery.

Conclusion: Mesenchymal stem cells and hematopoietic stem cells in the bone marrow are not involved in the regeneration of the periodontium. Cells that migrated from the residual periodontal ligament regenerated new alveolar bone at the early stage, and the regeneration around the dentin in the cavity was later than in other parts.

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The periodontal ligament plays supportive functions in the tooth, maintaining homeostasis and repair of damaged periodontium. The periodontal ligament consists of many synthetic cells, fibroblasts, osteoblasts and cementoblasts, which are important in repair of the periodontal ligament. Additionally, there have been many

reports that progenitor cells, which have the ability to differentiate into functional cells, are located near blood vessels in the periodontal ligament. These progenitor cells are able to differentiate into fibroblasts, osteoblasts and cementoblasts (1–3). Recently, cells expressing markers of bone marrow mesenchymal stem cells were isolated from the periodontal ligament by fluorescence-activated cell sorting, and some studies have reported that periodontal ligament cells include a population of multipotent stem cells capable of forming cementum and periodontal tissues (4,5). Moreover, bone marrow mesenchymal stem cells derived from the bone marrow have been clinically applied for transplants to damaged periodontium for regeneration and those cells were able to differentiate into specific periodontal tissue cells (6,7).

On the other hand, it is known that stem cells are less mature than progenitor cells and have the ability to differentiate into multiple types of cells as well as to self-renew and maintain their multipotential capacity, and exist in various tissues. In particular, bone marrow stem cells have similar properties to embryonic stem cells and they can be classified as bone marrow mesenchymal stem cells *and* bone marrow hematopoietic stem cells. However, what relationship they have, or what similarity or difference exists among the two subsets, is unknown (8). Bone marrow mesenchymal stem cells express a specific pattern of adhesion molecules, including CD29, CD44, CD71, CD90, CD105, CD166, SH2, SH3 and SH4, but do not express CD34 because they have been characterized phenotypically as nonhematopoietic cells (9–11). Bone marrow mesenchymal stem cells might be precursors of different types of mesenchymal cells such as chondrocytes, osteoblasts, adipocytes and myoblasts (9,10,12). Furthermore, circulating bone marrow mesenchymal stem cells can migrate to injured sites and can differentiate into fibroblasts and alveolar epithelial cells (13). Bone marrow mesenchymal stem cells cultured *in vitro* and transplanted into wound areas differentiate into blood vessels, hair follicles and sebaceous glands (14). On the other hand, bone marrow hematopoietic stem cells express a specific pattern of adhesion molecules, such as CD34 and c-KIT (15). Many studies have reported that bone marrow hematopoietic stem cells might differentiate into endothelial cells, hematopoietic cells and possibly neurons, fibroblasts and muscle cells (16–26). Bone marrow hematopoietic stem cells also migrate to remodeling glomerular and tubular epithelial phenotypes in the kidney (27–29). Therefore, these stem cells also migrate into wound areas from the blood and differentiate into several cell types to maintain various actions.

However, these stem cells or progenitor cells are still not completely understood and whether they are involved in the normal would healing of the periodontium is unknown. The purpose of this study was to identify stem cells or progenitor cells in the periodontal ligament and to investigate their behavior (using immunohistochemical methods) in the periodontal ligament during wound healing of bone defects created experimentally in the alveolar process.

Material and methods

This study was conducted in compliance with the Guidelines for the Treatment of Experimental Animals at Tokyo Dental College.

Twenty-five adult male Sprague-Dawley rats, each weighing about 200 g, were used in this study. Rats were anesthetized using 0.5 mL of 2.5% Ravonal® (Tanabe Seiyaku, Osaka, Japan). A skin incision was made along the inferior border of the mandible using a surgical knife and the anterior portion of the masseter muscle was reflected to expose the lateral surface of the alveolar bone overlying the mesial root of the first molar. Thereafter, an intradentinal cavity, $\approx$1 mm in diameter, was made using a round dental burr cooled with phosphate-buffered saline, pH 7.2. The debris was washed out with phosphate-buffered saline, and the masseter muscle and skin were sutured using a 3-0 silk strand. The animals were killed with an endoceliac injected overdose of thiopental, 1, 3, 5, 7 and 14 d after the operation. The mandible was removed, fixed in 10% formaldehyde for 24 h and then decalcified for 48 h with 10% formic acid buffered. The specimens were dehydrated in ethanol before being embedded in paraffin. Paraffin sections, $\approx$4 μm in thickness, were cut mesiodistally using a rotary thin-sectioning instrument. For light microscopic observations, paraffin sections were stained with hematoxylin and eosin.

Immunohistochemistry

Paraffin sections were deparaffinized with xylol and incubated in 3% hydrogen peroxide with methanol for 13 min at room temperature to block endogenous peroxidase activity. For antigen retrieval, sections were treated with 3% bovine serum albumin or 10% goat serum for 30 min at room temperature. Anti-CD44s (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), diluted 1 : 250, and anti-Cbfa-1 (supplied by Dr Sasaguri, Department of Orthodontics, Kanagawa Dental College, Japan), diluted 1 : 100, were used as primary antibodies. The sections were incubated at room temperature for 60 min and then were incubated with a biotinylated secondary antibody, NICHIREI-Histofine simple-stain MAX-PO® (Nichirei, Tokyo, Japan), for 30 min at room temperature. Thereafter, the sections were rinsed with phosphate-buffered saline and stained with NICHIREI-Histofine simple-stain Diaminobenzidine® (Nichirei) and counterstained with hematoxylin.

Anti-CD34 (Santa Cruz Biotechnology, Inc.), diluted 1 : 100, anti-c-KIT (Ventana, Tucson, AZ, USA), not diluted, STRO-1 (R & D Systems, Inc., Minneapolis, MN, USA), diluted 1 : 200, and anti-PCNA (Dako, Glostrup, Denmark), diluted 1 : 200, were also used as primary antibodies. After deparaffinization, the sections were microwaved for 20 min at 60°C for antigen retrieval. The sections were then incubated with those antibodies at 37°C for 32 min. The immunoperoxidase (avidin–biotin–peroxidase complex)

technique was performed using an automatic immunostaining device (Ventana NX System; Ventana, Tucson, AZ, USA/Tokyo, Japan) and Ventana kits, followed by counterstaining with hematoxylin.

Immunohistochemistry for 5-bromo-2-deoxyuridine incorporated *in vivo* in rat periodontal ligament

Ten rats were given a single intraperitoneal injection of 400 mg/kg of 5-bromo-2-deoxyuridine (Invitrogen™, Carlsbad, CA, USA) 1 d after the operation and were killed 2, 4 and 6 d later. Tissue samples were resected and fixed in 10% formaldehyde for 24 h and were then decalcified in 10% EDTA (pH 7.4) for 14 d. Paraffin sections were cut ≈4 μm in thickness, deparaffinized with xylol and then incubated in 2 N HCl for 30 min to denature the DNA. They were then incubated in 3% hydrogen peroxide with methanol for 13 min to block endogenous peroxidase activity. Anti-5-bromo-2-deoxyuridine (Abcam®, Cambridge, UK), diluted 1 : 300, was used as the primary antibody for 120 min. Immunostaining was carried out in the same manner described above.

Morphometric analysis

The upper and lower marginal lines of the cavity were drawn and divided into five areas: dentin cavity; periodontal ligament cavity; alveolar bone cavity; crown-side periodontal ligament and apical-side periodontal ligament (Fig. 1).

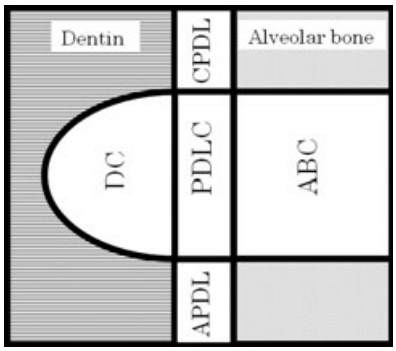

Fig. 1. Diagrammatic illustration of cavities in the first molar. ABC, alveolar bone cavity; APDL, apical-side periodontal ligament; CPDL, crown-side periodontal ligament; DC, dentin cavity; PDLC, periodontal ligament cavity.

Ratio of PCNA- and Cbfa-1-positive cells (score)

The total numbers of PCNA- and Cbfa-1-positive cells were calculated in

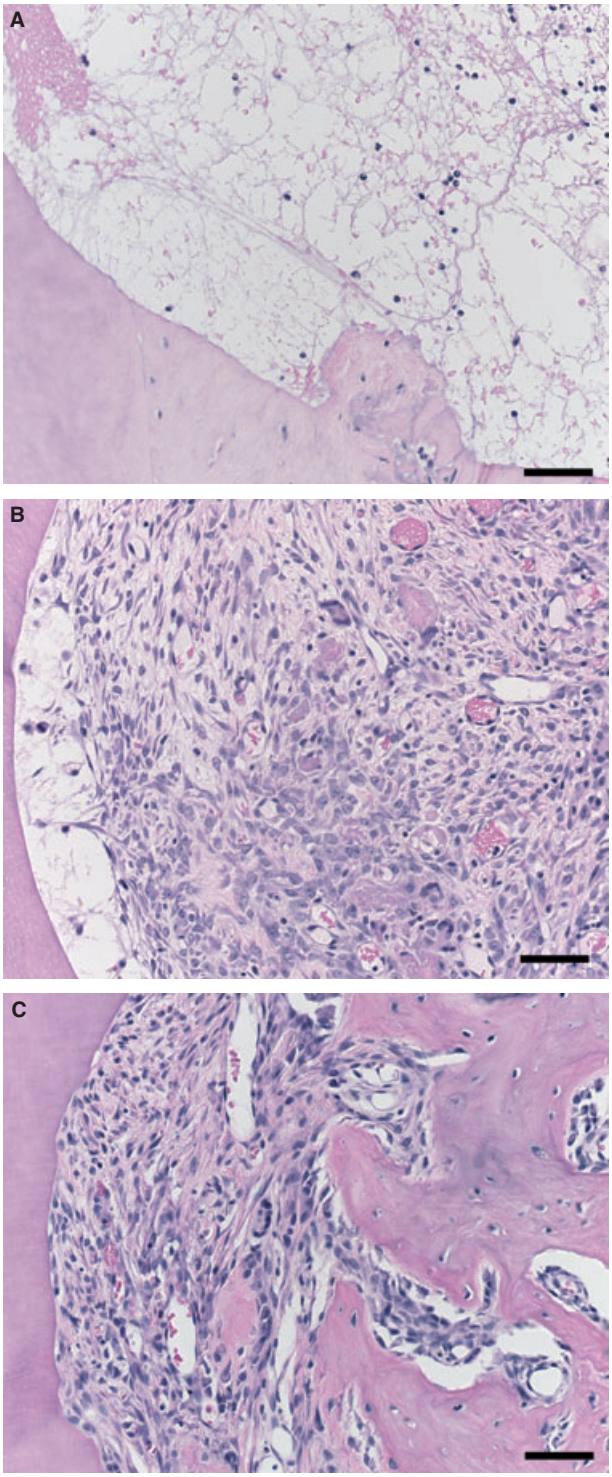

Fig. 2. Formation of cavity after surgery at 1 d (A), 5 d (B) and 14 d (C) in specimens stained with hematoxylin and eosin. The cavity was filled with fibrin and a few inflammatory cells (A). Many fibroblast-like cells filled the cavity (B). Newly formed periodontal ligament was observed in the cavity (C). Original magnification ×200; bar, 50 μm.

the immunohistochemically stained sections.

PCNA and Cbfa-1 scores were calculated for each of the 5 areas described above, according to the following equation: score = number of positive cells/10^4 μm^2.

Results are expressed as the mean PCNA and Cbfa-1 score ± standard deviation.

One-way analysis of variance and multiple comparison tests (Scheffé) were used to analyze the data for PCNA and Cbfa-1.

Results

Histological observations

Control — A few CD34-, c-KIT- and CD44s-positive cells were observed in cells of the alveolar bone marrow, but not in cells of the periodontal ligament. Cbfa-1-positive cells were observed in cells at the surface of the alveolar bone and around blood vessels in the apical-side periodontal ligament.

Experimental — One day after the operation, the cavity was filled with fibrin, and a few inflammatory cells, mainly neutrophils, were observed (Fig. 2A). Many STRO-1-, PCNA- and Cbfa-1-positive cells were observed on the cut surface of the crown-side periodontal ligament and the apical-side periodontal ligament (Fig. 3C).

Three days after the operation, the amount of fibrin had decreased and fibroblast-like cells appeared along the root surface and on the alveolar bone at the dentin cavity, the periodontal ligament cavity and the alveolar bone cavity.

STRO-1-positive cells were observed in the dentin cavity. PCNA- and Cbfa-1-positive cells were mainly observed in the crown-side periodontal ligament, periodontal ligament cavity and apical-side periodontal ligament (Fig. 3A).

Five days after the operation, many fibloblast-like cells filled the cavity (Fig. 2B). CD44s-positive cells were found along the alveolar bone in the alveolar bone cavity (Fig. 4B). STRO-1-positive cells were observed diffusely in the dentin cavity and the periodontal ligament cavity. Numerous PCNA- and Cbfa-1-positive cells were observed to be present diffusely in the cavities.

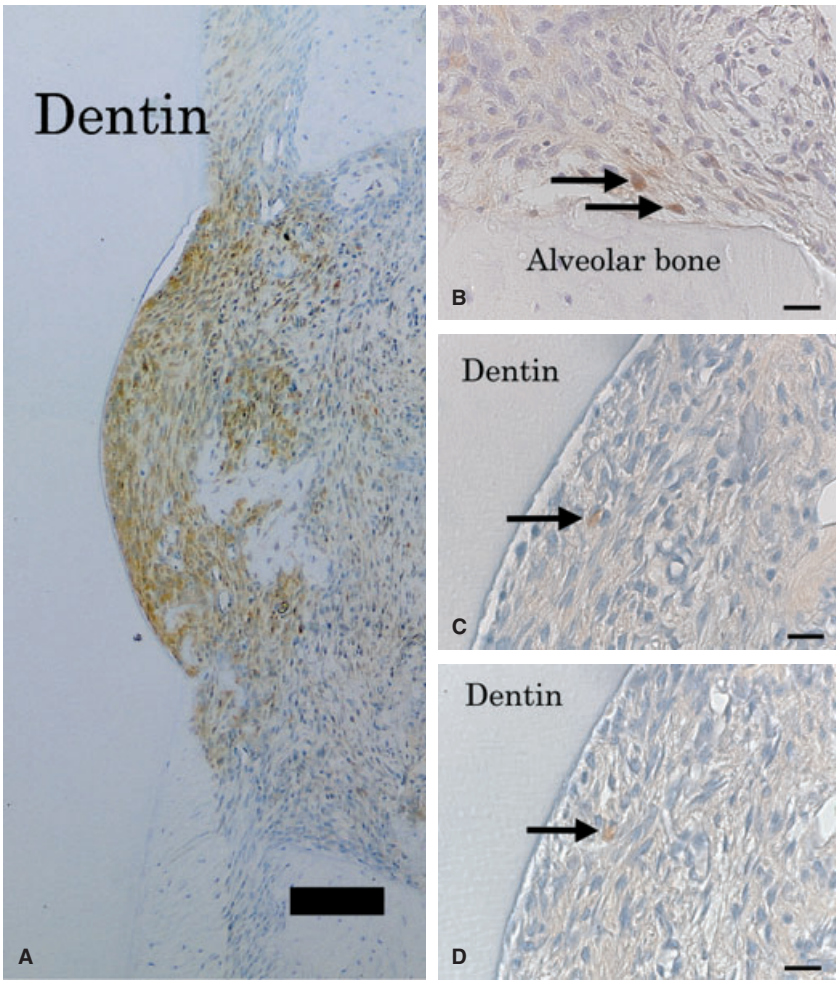

Fig. 3. Sections were stained with anti-STRO-1 (A), anti-CD44s (B), anti-CD34 (C), and anti-c-KIT (D). Many STRO-1-positive cells were observed along the dentin 7 d after the operation (A). CD44s-positive cells were found along the alveolar bone 5 d after the operation (B). CD34- and c-KIT-positive cells were found close to dentin 7 d after the operation (C,D). Original magnification ×50; bar, 50 μm (A). Original magnification ×400; bar, 20 μm (B–D).

Seven days after the operation, newly formed bone was observed in both the periodontal ligament cavity and the alveolar bone cavity. Cementoblast-like cells were arrayed along the dentin surface in the dentin cavity. CD34- and c-KIT-positive cells were found close to the dentin in the dentin cavity (Fig. 4C,D). CD44s-positive cells were not found in the dentin cavity. Many STRO-1-positive cells were observed in the dentin cavity and only a few in the alveolar bone cavity (Fig. 4A). Many PCNA- and Cbfa-1-positive cells were found close to the dentin in the dentin cavity (Fig. 3B,D).

Fourteen days after the operation, a thin, newly formed cementum was observed along the dentin surface in the dentin cavity (Fig. 2C). Newly formed periodontal ligament, the width of which was maintained constantly, was observed in the dentin cavity and the periodontal ligament cavity. PCNA-positive cells were observed mainly in the dentin cavity. Cbfa-1-positive cells were observed in the newly formed bone and blood vessels.

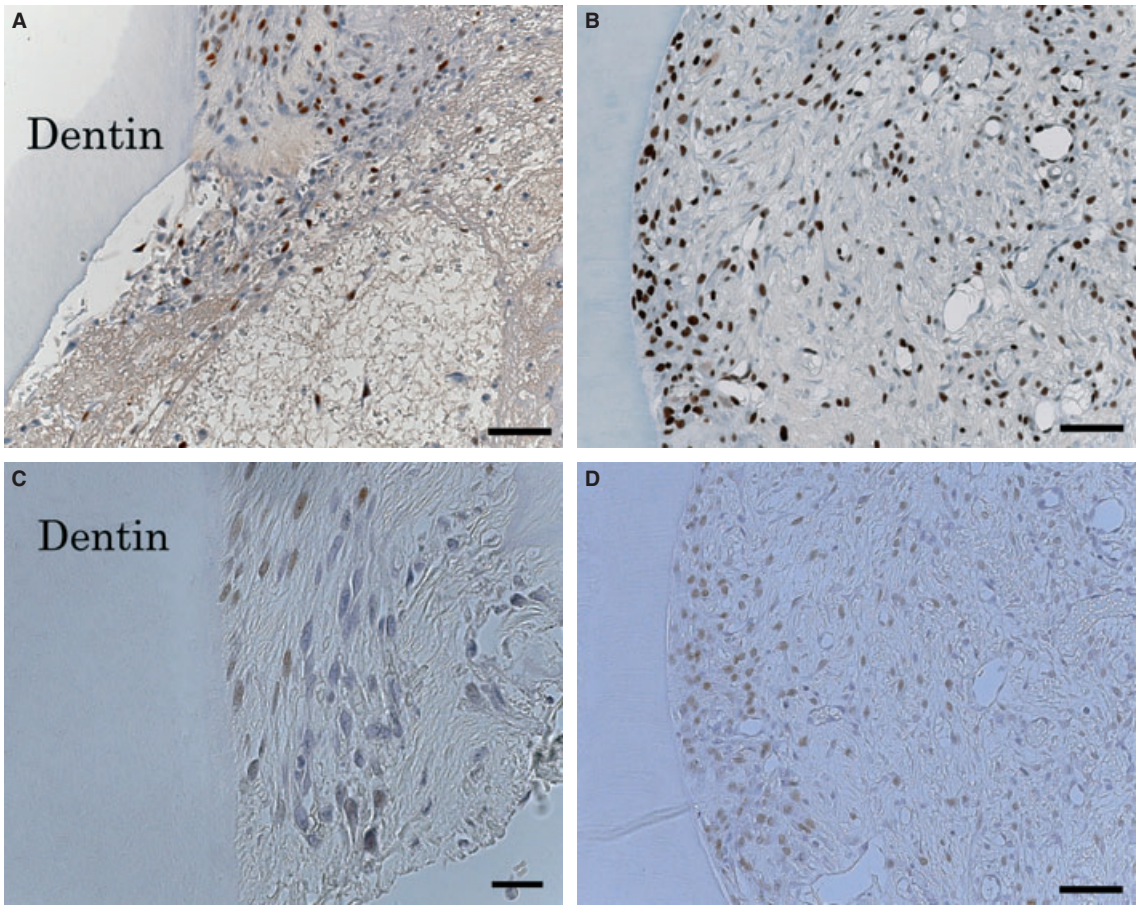

Fig. 4. Sections were stained with anti-PCNA (A,B) and anti-Cbfa-1 (C,D). PCNA-positive cells were observed in the residual periodontal ligament 3 d after the operation (A) and on the surface of dentin 7 d after the operation (B). Cbfa-1-positive cells were observed around blood vessels 1 d after the operation (C) and at the surface of dentin 7 d after the operation (D). Original magnification ×200; bar, 50 μm (A,B,D). Original magnification ×400; bar, 20 μm (C).

Observations following injection of 5-bromo-2-deoxyuridine

5-Bromo-2-deoxyuridine-positive cells were observed in the residual periodontal ligament, and a few 5-bromo -2-deoxyuridine-positive cells were observed in the apical-side periodontal ligament and the crown-side periodontal ligament 3 d after the operation (Fig. 5A).

A number of 5-bromo-2-deoxyuridine-positive cells were observed in the periodontal ligament cavity 5 d after the operation (Fig. 5B).

Osteoblasts, fibloblasts and endothelial cells located in the newly formed bone and blood vessels in the dentin cavity were 5-bromo-2-deoxyuridine positive 7 d after the operation (Fig. 5C).

Histomorphometric analysis

One day after the operation, PCNA scores in the crown-side periodontal ligament and the apical-side periodontal ligament were significantly higher than those in the other cavities ($p < 0.01$ and $p < 0.05$, respectively) (Fig. 6A). The Cbfa-1 scores in the crown-side periodontal ligament and the apical-side periodontal ligament were significantly higher than those in the other cavities ($p < 0.01$) (Fig. 7A).

Three days after the operation, the PCNA score in the periodontal ligament cavity was significantly higher than those in the dentin cavity and the alveolar bone cavity ($p < 0.05$) (Fig. 6B). The Cbfa-1 scores in the crown-side periodontal ligament and the apical-side periodontal ligament were significantly higher than those in the dentin cavity, the periodontal ligament cavity and the alveolar bone cavity ($p < 0.01$ and $p < 0.05$) (Fig. 7B).

Five days after the operation, the PCNA scores tended to increase; however, there were no significant differences among the cavities (Fig. 6C). The Cbfa-1 score in the dentin cavity was significantly lower than those in the other cavities ($p < 0.01$) (Fig. 7C).

Seven days after the operation, PCNA scores tended to decrease, although the PCNA score in the dentin cavity increased and was significantly higher than in the alveolar bone cavity ($p < 0.01$) (Fig. 6D). On the other hand, the Cbfa-1 score tended to increase and the Cbfa-1 score in the

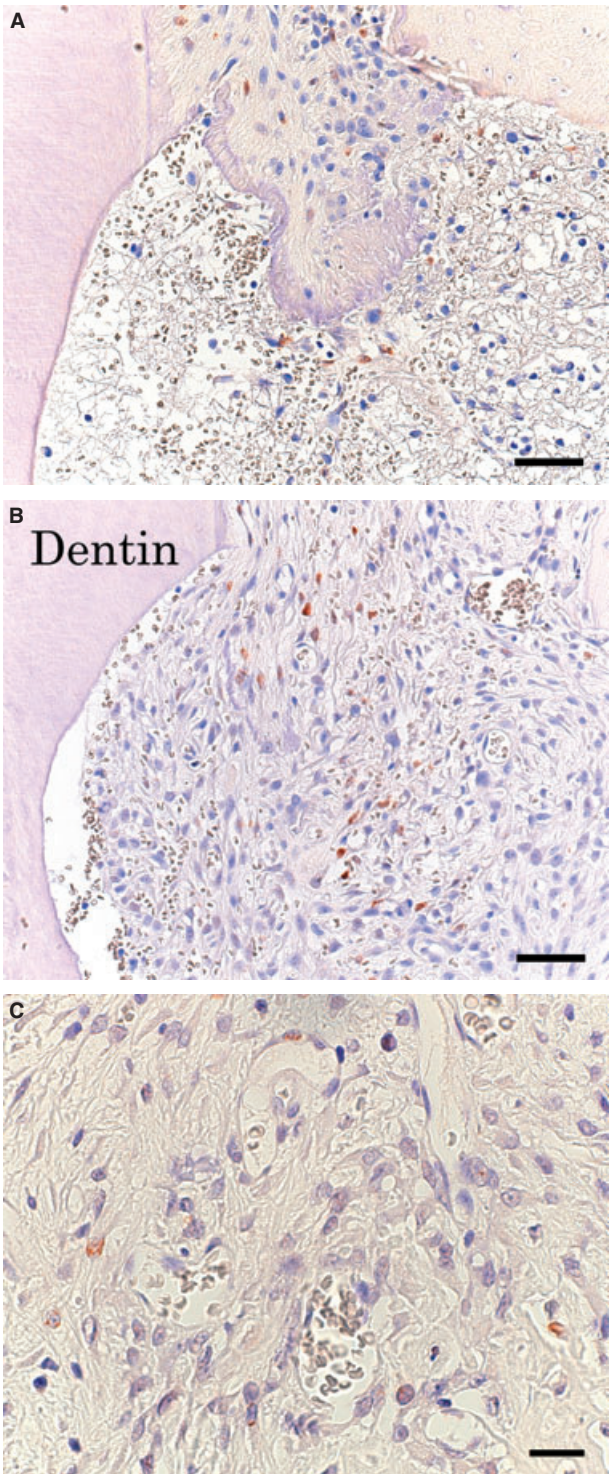

Fig. 5. When 5-bromo-2-deoxyuridine was injected 1 d after the operation, 5-bromo-2-deoxyuridine-labeled cells were observed in the upper periodontal ligament 3 d after the operation (A), in the middle cavity and residual periodontal ligament 5 d after the operation (B), and in the new alveolar bone 7 d after the operation (C). Original magnification ×200; bar, 50 μm (A,B). Original magnification ×400; bar, 20 μm (C).

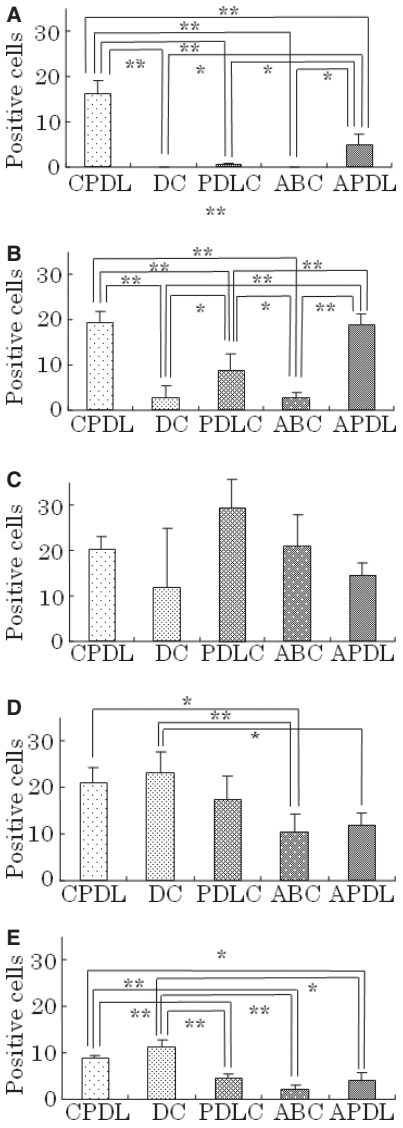

Fig. 6. The ratio of PCNA-positive cells were component PCNA scores in the crown-side periodontal ligament, dentin cavity, periodontal ligament cavity, alveolar bone cavity and apical-side periodontal ligament ($n = 5$) 1 d (A), 3 d (B), 5 d (C), 7 d (D) and 14 d (E) after surgery. *Significantly different ($p \leq 0.05$). ** Significantly different ($p \leq 0.01$). ABC, alveolar bone cavity; APDL, apical-side periodontal ligament; CPDL, crown-side periodontal ligament; DC, dentin cavity; PDLC, periodontal ligament cavity.

dentin cavity was significantly higher than in the alveolar bone cavity ($p < 0.01$) (Fig. 7D).

Fourteen days after the operation, the PCNA and Cbfa-1 scores tended to decrease overall. However, the PCNA score in the dentin cavity was significantly higher than the PCNA score in the periodontal ligament cavity, the alveolar bone cavity and the apical-side periodontal ligament ($p < 0.01$) (Fig. 6E). The Cbfa-1 scores were not

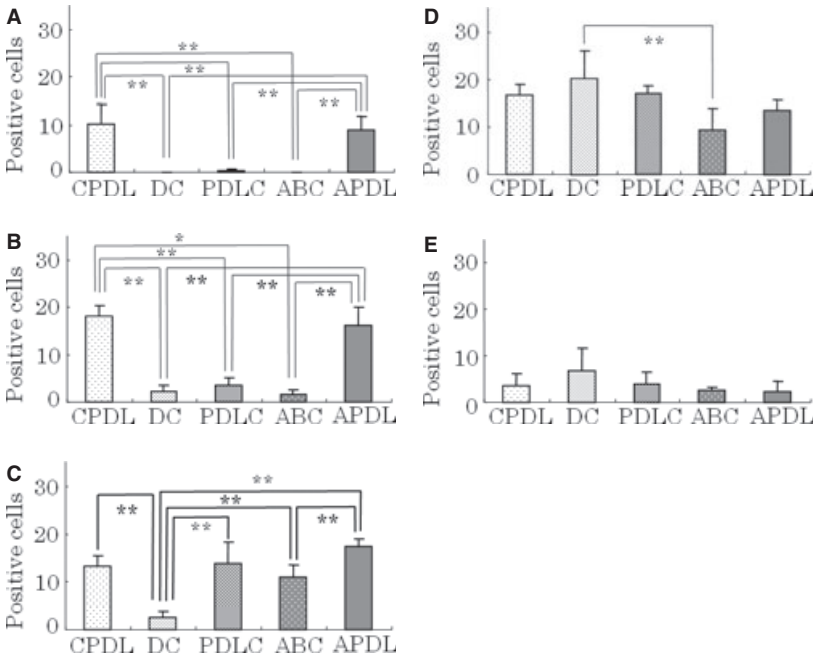

Fig. 7. The ratio of Cbfa-1 positive cells were component PCNA scores in the crown-side periodontal ligament, dentin cavity, periodontal ligament cavity, alveolar bone cavity and apical-side periodontal ligament ($n = 5$) 1 d (A), 3 d (B), 5 d (C), 7 d (D) and 14 d (E) after surgery. *Significantly different ($p \leq 0.05$). **Significantly different ($p \leq 0.01$). ABC, alveolar bone cavity; APDL, apical-side periodontal ligament; CPDL, crown-side periodontal ligament; DC, dentin cavity; PDLC, periodontal ligament cavity.

significantly different in any of the cavities (Fig. 7E).

Discussion

It is known that mesenchymal stem cells and hematopoietic stem cells exist in the bone marrow. Bone marrow mesenchymal stem cells have been recently reported to be able to migrate into skeletal and cardiac muscle and then to differentiate into skeletal and cardiac muscle cells (30–35). Bone marrow hematopoietic stem cells also migrate to remodeling glomerular and tubular epithelial phenotype cells in the kidney (19). However, bone marrow mesenchymal stem cells are present as a rare population of cells in the bone marrow, representing perhaps 0.001–0.01% of the nucleated cells (36). Bone marrow hematopoietic stem cells are also a rare population, present at ≈ 0.05– 0.1% in the bone marrow (37–39). Therefore, it is questionable whether these cells are involved in the regeneration of the periodontium during wound healing.

The periodontal ligament is a mesenchymal tissue consisting of fibroblasts, osteoblasts and cementoblasts, cells that are required for the regeneration of the periodontium. Recently, some human periodontal ligament cells have been reported to express the bone marrow mesenchymal stem cell markers, CD105 and CD166, as analyzed by fluorescence-activated cell sorting (4,5). Those cells should be capable of differentiating into cementoblastic/osteoblastic cells *in vitro* and of forming cementum/periodontal ligament-like tissues *in vivo* (4–6). In the present study, an antibody to CD44s was used to detect bone marrow mesenchymal stem cells, and antibodies to CD34 and c-KIT were used to detect bone marrow hematopoietic stem cells. A few CD44s-, CD34- and c-KIT-positive cells were observed in the alveolar bone marrow, but not in the periodontal ligament. Only a few CD44s-positive cells were found along the alveolar bone at 5 d, and a few CD34- and c-KIT-positive cells were found close to the dentin at 7 d, after surgery (Fig. 3B–D). This suggests that bone marrow mesenchymal stem cells and bone marrow hematopoietic stem cells do not exist in the periodontal ligament and that these cells from the bone marrow might not be involved in the regeneration of periodontium. Chen *et al*. reported that putative stem cells for which STRO-1, CD146 CD44 positive cells in the periodontal ligament were associated enhanced the number of these cells by the inflammatory (40).

On the other hand, Gould *et al*. reported that progenitor cells located near blood vessels in the periodontal ligament are capable of differentiating into fibroblasts, osteoblasts and cementoblasts during wound healing of the periodontium. Cbfa-1 is a transcription factor activated at the onset of osteogenesis and is considered to be a robust marker of osteogenic commitment (41–43). STRO-1 is known as an early marker of different bone marrow mesenchymal stem cells and pre-osteogenic populations, which infers a possible perivascular niche for these cell populations *in situ*, and expression of STRO-1 is progressively lost after cell proliferation and differentiation into mature osteoblasts *in vitro* (44–47). Therefore, Cbfa-1- and STRO-1-positive cells might be progenitor cells. In this study, Cbfa-1-positive cells increased in the cavity up to 7 d after the operation and STOR-1-positive cells were mainly observed along the dentin surface 7 d after the operation (Figs 3A and 4D). It is suggested that these progenitor cells mainly participate in the regeneration of periodontal ligament in the dentin cavity.

5-Bromo-2-deoxyuridine is incorporated into nuclear DNA during the S phase of the cell cycle and is used to label proliferating cells (48). PCNA is a 36-kDa acidic nonhistone nuclear protein that functions as an auxiliary protein for DNA delta polymerase and is an absolute requirement for DNA synthesis. Therefore, 5-bromo-2-deoxyuridine-positive cells, which may be progenitor cells, were observed in the periodontal ligament and had migrated into the wound area. The

PCNA scores tended to increase from the cut edge of the residual periodontal ligament to the cavity at early stages and then increased in the overall cavity (Fig. 6). 5-Bromo-2-deoxyuridine-labeled cells were observed around the cut edge of the residual periodontal ligament at 3 d and then were observed at the center of the cavity at 5 d (Fig. 5A,B). Finally, these cells were observed in the newly formed alveolar bone and cells of blood vessels (Fig. 5C). These observations suggest that these progenitor cells located in the residual periodontal ligament migrate and differentiate into osteoblasts and are involved in the regeneration of the periodontium.

On the other hand, the PCNA scores increased in the dentin cavity later than in other cavities (Fig. 6). The Cbfa-1 scores tended to increase in all cavities in a manner similar to the PCNA scores (Figs 6 and 7). However, the Cbfa-1 scores were lower than the PCNA scores. PCNA-positive cells might consist of osteoblasts and other cells, such as fibroblasts. However, the Cbfa-1 scores were much lower than PCNA scores only in the dentin cavity at 5 d (Fig. 7C). Pratap *et al*. reported that Cbfa-1 may directly or indirectly regulate proliferation by (de)sensitizing cells to bone-related external stimuli (49). Therefore, osteoblasts might start to differentiate in the periodontal ligament cavity at 5 d. This suggests that osteoblasts involved in the regeneration of the alveolar bone differentiate earlier than other cells involved in the regeneration of the periodontium.

From these results, we conclude that bone marrow mesenchymal stem cells and bone marrow hematopoietic stem cells in the bone marrow may not be involved in the regeneration of the periodontium. Cells that migrate from the residual periodontal ligament regenerate new alveolar bone at an early stage and the regeneration around the dentin in the cavity occurs later than in other parts of the periodontal ligament.

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