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Author(s)
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The behavior of stem cells and progenitor cells in the periodontal ligament during wound healing using immunohistochemical methods.

Suguru Ohta¹, Satoru Yamada¹, Keniti Matuzaka², Takasi Inoue²*

¹ Department of Periodontology, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba 261-8502, Japan

² Department of Clinical Pathophysiology, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba, 261-8502, Japan

Running title:

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*Corresponding author: Professor Takashi Inoue,
Department of Clinical Pathophysiology, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba, 261-8502, Japan

TEL: +81-43-270-3582

FAX: +81-43-270-3583

e-mail: inoue@tdc.ac.jp
Background and Objective: The aim of this study was to identify stem cells or progenitor cells in the periodontal ligament (PDL) and investigate their behavior during wound healing of bone defects created experimentally in the alveolar process.

Material and Methods: Intrabulbar cavities were created in the mesial root of the first molar of 25 adult male rats which were sacrificed at 1, 3, 5, 7 and 14 days after the surgery. At each time point, sections were stained immunohistochemically for CD44s (standard), CD34, c-KIT, PCNA, cbfa-1 and BrdU using primary antibodies. For morphometric analysis, the ratios of cbfa-1-and PCNA-positive cells were calculated from the total numbers of positive cells / 10^4 µm² in the cavities.

Results: BrdU positive cells were observed in the PDL 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 PDL. CD44s positive cells were only observed in the alveolar bone cavity at 5 days. CD34 and c-KIT positive cells were only observed in the dentin cavity at 7 days. Cbfa-1 and PCNA scores tended to increase at 7 days after the operation.

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

Key words: stem cells, progenitor cells, wound healing, periodontal ligament
**Introduction**

The periodontal ligament (PDL) plays supportive functions in the tooth, maintaining homeostasis and repair of damaged periodontium. The PDL consists of many synthetic cells; fibroblasts, osteoblasts and cementoblasts which are important to repair the PDL. Additionally, there have been many reports that progenitor cells, which have the ability to differentiate into functional cells, located near blood vessels in the PDL. These progenitor cells are able to differentiate into fibroblasts, osteoblasts and cementoblasts (1-3). Recently, cells expressing markers of bone marrow mesenchymal stem cells (BMMSCs) were isolated from the PDL by fluorescence activated cell sorting and some studies reported that PDL cells include a population of multipotent stem cells capable of forming cementum and periodontal tissues (4, 5). Moreover, BMMSCs 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, exist in various tissues. In particular, bone marrow stem cells have similar properties to embryonic stem cells and they can be classified as BMMSCs and bone marrow hematopoietic stem cells (BMHSCs). However, what relationship they have or what similarity or difference exists among the two subsets is unknown (8). BMMSCs express a specific pattern of adhesion molecules, including CD29, CD44, CD71, CD90, CD105, CD166, SH2, SH3
and SH4, but do not express CD34 since they have been characterized phenotypically as non-hematopoietic cells (9-11). BMMSCs might be precursors of different types of mesenchymal cells such as chondrocytes, osteoblasts, adipocytes and myoblasts (9,10,12). Furthermore, circulating BMMSCs can migrate to injured sites and can differentiate into fibroblasts and alveolar epithelial cells (13). BMMSCs cultured in vitro and transplanted into wound areas differentiate into blood vessels, hair follicles and sebaceous glands (14). On the other hand, BMHSCs express a specific pattern of adhesion molecules, such as CD34 and c-KIT (15). Many studies have reported that BMHSCs might differentiate into endothelial cells, hematopoietic cells, and possibly neurons, fibroblasts and muscle cells (16-26). BMHSCs 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 wound healing of the periodontium is unknown. The purpose of this study was to identify stem cells or progenitor cells in the PDL and investigate their behavior in the PDL during wound healing of bone defects created experimentally in the alveolar process using immunohistochemical methods.
Materials 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 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, approximately 1 mm in diameter, was made using a round dental burr cooled with phosphate buffered saline (PBS, pH 7.2). The debris was washed out with PBS and the masseter muscle and skin were sutured using a 3-0 silk strand. The animals were euthanized with an endoceliac injected overdose of thiopental at 1, 3, 5, 7 and 14 days after the operation. The mandible was removed and was fixed in 10% formaldehyde for 24 h and then was decalcified with 10% formic acid buffered for 48 h. The specimens were dehydrated in ethanol before being embedded in paraffin. Paraffin sections, approximately 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 (HE).
Immunohistochemistry

Paraffin sections were deparaffinized with xylol and were 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 (BSA) or 10% goat serum for 30 min at room temperature. Anti-cluster of differentiation 44 standard (CD44s: Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) diluted at 1:250 and anti-core binding factor a-1 (cbfa-1: supplied by Dr. Sasaguri, Department of Orthodontics, Kanagawa Dental College, Japan) diluted at 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 PBS and were stained with NICHIREI-Histofine simple-stain DAB® (NICHIREI, Tokyo, Japan) and counterstained with Hematoxylin.

Anti-cluster of differentiation 34 (CD34: Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) diluted at 1:100, anti-c-KIT (VENTANA, Tucson, AZ, USA) not diluted, STRO-1 (R&D Systems, Inc., Minneapolis, MN, USA) diluted at 1:200, and anti-proliferation cell nuclear antigen: PCNA (DAKO, Glostrup, Denmark) diluted at 1:200 were also used as primary antibodies. After deparaffinizing, 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, Tucson, AZ, USA/Tokyo, Japan) and Ventana kits followed by counterstaining with Hematoxylin.

**Immunohistochemistry for BrdU incorporated *in vivo* in rat PDL**

Ten rats were given a single intraperitoneal injection of 400 mg/kg BrdU (Invitrogen™, Carlsbad, CA, USA) 1 day after the operation and were sacrificed 2, 4 and 6 days later. Tissue samples were resected and fixed in 10% formaldehyde for 24 hr and were then decalcified in 10% EDTA (pH 7.4) for 14 days. Paraffin sections were cut approximately 4 µm in thickness and were deparaffinized with xylol and then incubated in 2N 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-BrdU (Abcam®, Cambridge, UK) diluted at 1:300 was used as 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 were divided into five areas; dentin, dentin cavity (DC), periodontal ligament cavity (PDLC) and alveolar bone cavity (ABC) (Fig 1). The remaining PDL was named the crown side periodontal ligament (CPDL) and the apical side periodontal ligament (APDL) (Fig 1).
Ratio of PCNA and cbfa-1 positive cells (score)
The total numbers of PCNA and cbfa-1 positive cells were calculated in the
immunohistochemically stained sections.

  PCNA and cbfa-1 scores were calculated for each of the 5 parts described
above.

  Score = number of positive cells / $10^4 \mu m^2$

Results are expressed as the mean PCNA and cbfa-1 score ± SD

One-way analysis of variance (ANOVA) and multiple comparison test
(Scheffe) was 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 PDL. Cbfa-1 positive cells were observed in cells at the surface of the alveolar bone and around blood vessels in the APDL.

Experimental

At 1 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 CPDL and the APDL (Fig. 4c).

At 3 days after the operation, the amount of fibrin had decreased and fibroblast-like cells appeared along the root surface and the alveolar bone at the DC, PDLC and ABC. STRO-1 positive cells were observed in the DC. PCNA and cbfa-1 positive cells were mainly observed in the CPDL, PDLC and APDL (Fig. 4a).

At 5 days after the operation, many fibroblast-like cells filled the cavity (Fig. 2b). CD44s positive cells were found along the alveolar bone in the ABC (Fig. 3b). STRO-1 positive cells were observed diffusely in the DC and the PDLC. Numerous PCNA and cbfa-1 positive cells were diffusely observed in the
cavities.

At 7 days after the operation, newly formed bone was observed in both the PDLC and the ABC. Cementoblast-like cells were arrayed along the dentin surface in the DC. CD34 and c-KIT positive cells were found close to the dentin in the DC (Fig. 3c, d). CD44s positive cells were not found in the DC. Many STRO-1 positive cells were observed in the DC and only a few in the ABC (Fig. 3a). Many PCNA and cbfa-1 positive cells were found close to the dentin in the DC (Fig. 4b, d).

At 14 days after the operation, a thin newly-formed cementum was observed along the dentin surface in the DC (Fig. 2c). Newly-formed PDL, the width of which was maintained constantly, was observed in the DC and the PDLC. PCNA positive cells were observed mainly in the DC. Cbfa-1 positive cells were observed in the newly formed bone and blood vessels.

Observations following BrdU injection
BrdU positive cells were observed in the residual PDL and a few BrdU positive cells were observed in the APDL and the CPDL at 3 days after the operation (Fig. 5a).
A number of BrdU positive cells were observed in the PDLC at 5 days after the operation (Fig. 5b).
Osteoblasts, fibroblasts and endothelial cells located in the newly formed bone and blood vessels in the DC were BrdU positive at 7 days after the
Histomorphometric analysis

At 1 day after the operation, PCNA scores in the CPDL and the APDL 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 CPDL and the APDL were significantly higher than those in the other cavities (P<0.01) (Fig 7a).

At 3 days after the operation, the PCNA score in the PDLC was significantly higher than those in the DC and the ABC (P<0.05) (Fig 6b). The cbfa-1 scores in the CPDL and the APDL were significantly higher than those in the DC, PDLC and ABC (P<0.01 and P<0.05) (Fig 7b).

At 5 days after the operation, PCNA scores tended to increase, however there were no significant differences among the cavities (Fig 6c). The cbfa-1 score in the DC was significantly lower than those in the other cavities (P<0.01) (Fig 7c).

At 7 days after the operation, PCNA scores tended to decrease, although the PCNA score in the DC increased and was significantly higher than in the ABC (P<0.01) (Fig 6d). On the other hand, the cbfa-1 score tended to increase, the cbfa-1 score in the DC was significantly higher than in the ABC (P<0.01) (Fig 7d).
At 14 days after the operation, the PCNA and cbfa-1 scores tended to decrease overall. However, the PCNA score in the DC was significantly higher than in the PDLC, the ABC and the APDL (P<0.01) (Fig 6e). The cbfa-1 scores were not significantly different in any of the cavities (Fig 7e).
Discussion

It is known that MSCs and HSCs exist in the bone marrow. BMMSCs 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). BMHSCs also migrate to remodeling glomerular and tubular epithelial phenotype cells in the kidney (19). However, BMMSCs are present as a rare population of cells in the bone marrow, representing perhaps 0.001% to 0.01% of the nucleated cells (36). BMHSCs are also a rare population, approximately 0.05% to 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 PDL is a mesenchymal tissue consisting of fibroblasts, osteoblasts and cementoblasts, cells that are required for the regeneration of the periodontium. Recently, some human PDL cells have been reported to express the BMMSC 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/PDL-like tissues in vivo (4-6). In this study, an antibody to CD44s was used to detect BMMSCs and antibodies to CD34 and c-KIT were used to detect BMHSCs. A few CD44s, CD34 and c-KIT positive cells were observed in the alveolar bone marrow, but not in the PDL. Only a few CD44s positive cells were found along the alveolar bone at 5 days and a few CD34 and c-KIT positive cells were found close to the dentin at 7 days (Fig 3b, c and d). This suggests that BMMSCs and BMHSCs do not exist in the PDL and that these
cells from the bone marrow might not be involved in the regeneration of periodontium. Recently Chen SC et al reported that putative stem cells which STRO-1, CD146 CD44 positive cells in the PDL were associated to enhance 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 PDL 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 a robust marker of osteogenic commitment (41-43). STRO-1 is known as an early marker of different BMMSCs and preosteogenic 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 days after the operation and STOR-1 positive cells were mainly observed along the dentin surface at 7 days after the operation (Fig 4d and Fig 3a). It is suggested that these progenitor cells are mainly participated in the regeneration of PDL in the dentin cavity.

BrdU 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 kD acidic non-histone nuclear protein that functions as an auxiliary protein for DNA delta polymerase and is an absolute requirement for DNA synthesis. Therefore, BrdU positive cells, which may be progenitor cells, were observed
in the PDL and had migrated into the wound area. PCNA scores tended to increase from the cut edge of the residual PDL to the cavity at early stages and then increased in the overall cavity (Fig 6). BrdU labeled cells were observed around the cut edge of the residual PDL at 3 days and then were observed at the center of the cavity at 5 days (Fig 5a and 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 PDL migrate and differentiate into osteoblasts and are involved in the regeneration of the periodontium.

On the other hand, PCNA scores increased in the DC later than in other cavities (Fig 6). Cbfa-1 scores tended to increase in all cavities similar to the PCNA scores (Fig 6 and Fig 7). However, the cbfa-1 scores were lower than the PCNA score. PCNA positive cells might consist of osteoblasts and other cells such as fibroblasts. However, cbfa-1 scores were much lower than PCNA scores only in the DC at 5 days (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 PDLC at 5 days. 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 BMMSCs and BMHSCs in the bone marrow may not be involved in the regeneration of the periodontium. Cells which migrate from the residual PDL regenerate new alveolar bone at an early stage and the regeneration around the dentin in the cavity occurs later
than other parts as the PDL.

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

Fig. 2.
Formation of cavity after operation at 1 day (a), 5 days (b) and 14 days (c) in specimens stained with hematoxylin and eosin. The cavity was filled with fibrin and a few inflammatory cells (a). Many fibroblasts-like cells were filled the cavity (b). Newly formed PDL was observed in the cavity (c).
(Original magnification: (a-c) x 200. Bar: (a-c) 50 μm).

Fig. 3.
Sections were stained with anti- STRO-1(a), anti-CD44s (b), anti-CD34 (c), and anti-c-KIT (d) antibodies. Many STRO-1 positive cells were observed along the dentin at 7 days after the operation (a). CD44s positive cells were found along the alveolar bone at 5 days after the operation (b). CD34 and c-KIT positive cells were found close to dentin at 7 days after the operation (c, d).
(Original magnification: (a) x 50, (b-d) x 400. Bar: (a) 50 μm; (b-d) 20 μm).

Fig. 4.
Sections were stained with anti- PCNA (a-b) and anti- cbfa-1 (c-d) antibodies.
PCNA positive cells were observed in the residual PDL at 3 days after the operation (a) and surface of dentin at 7 days after the operation (b).
Cbfa-1 positive cells were observed around blood vessels at 1 day after the operation (c) and at the surface of dentin at 7 days after the operation (d).
(Original magnification: (a, b, d) x 200, (c) x 400. Bar: (a, b, d) 50 μm; (c) 20 μm).

Fig. 5.
When BrdU was injected at 1 day after the operation, BrdU labeled cells were observed in the upper PDL at 3 day after the operation (a), in the middle cavity and residual PDL at 5 days after the operation (b), but in the new alveolar bone at 7 days after the operation (c).
(Original magnification: (a, b) x 200, (c) x 400. Bar: (a, b) 50 μm; (c) 20 μm.)
Fig. 6.
The ratio of PCNA positive cells were component PCNA scores in cavities UPDL, DC, PDLC, ABC and LPDL (n=5) at the each of 1 day (a), 3 days (b), 5 days (c), 7 days (d) and 14 days (e). *Significantly different (p<0.05). ** Significantly different (p<0.01)

Fig. 7.
The ratio of cbfa-1 positive cells were component PCNA scores in cavities UPDL, DC, PDLC, ABC and LPDL (n=5) at the each of 1 day (a), 3 days (b), 5 days (c), 7 days (d) and 14 days (e). *Significantly different (p<0.05). ** Significantly different (p<0.01)
Fig. 1

Dentin

CPDL

Alveolar bone

DC

PDLC

ABC

APDL
Fig. 3

Dentin

Alveolar bone

Dentin

Dentin
Fig. 4
Fig. 6
Fig. 7