

学位論文

論文題目

Histological Evaluation of Periodontal Ligament in
Response to Orthodontic Mechanical Stress in Mice

(歯科矯正学的メカニカルストレスによるマウス
歯周組織改造における細胞動態)

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松本歯科大学大学院歯学独立研究科博士(歯学)学位申請論文

Title

Histological Evaluation of Periodontal Ligament in Response
to Orthodontic Mechanical Stress in Mice

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要旨

【目的】歯周組織改造時の細胞の動態を包括的に明らかにするため、歯科矯正学的メカニカルストレスを負荷し、歯周組織の細胞数の経時的変化を測定し、細胞の供給源を特定と骨髄由来細胞が歯周組織構成細胞に分化するのかを追究した。

【方法】8週齢の ddY 系雄性マウス 10 匹を用い、Waldo 法によって上顎第一、第二臼歯間にラバーダムを挿入、ストレスを 3 時間負荷した。負荷を解除して 1 週間まで病理組織学的に検討し、当該部歯周組織の圧迫側と牽引側における歯周組織内の細胞数を計測した。7週齢の GFP トランスジェニックマウスから骨髄移植を受けた GFP マウス 10 匹を用い、同様の方法でストレスを 3 時間負荷した直後から 6 か月経過した標本に各種免疫染色を施し、GFP 陽性の移植骨髄由来の細胞の発現の様相を観察し、細胞分化の様相を明らかにした。なお、GFP マウス由来骨髄由来細胞の調整は、GFP トランスジェニック動物をエーテル麻酔化にて屠殺し大腿骨を摘出し、骨髄細胞を採取した骨髄細胞を抗生剤を含む培地で洗浄後、HBBS に置換、GFP マウスと同系の 7 週齢雄マウスに X 線照射を行った後、尾静脈から骨髄細胞を移植した。対照は、無処置の同種歯根膜部を使用した。免疫染色は、抗 GFP 抗体に Anti-GFP antibody-ChIP Grade ab290 を用い、5000 倍に希釈し、4℃で overnight 反応させた。二次抗体として、抗ウサギポリクローナル抗体と反応させ、PBS で洗浄後、発色は DAB により行った。GFP に関しては病理組織学的検討と同様の方法で、陽性細胞占有率で計測した。

蛍光二重染色には、抗 GFP 抗体はヤギポリクローナル抗体を用い、抗 CD31 抗体、抗 CD68 抗体、および Runx2 抗体を用いて、GFP と組み合わせで行った。一次抗体を 4℃で overnight 反応させた。二次抗体として Alexa Fluor®568 Labeled Donkey Anti-Goat IgG Antibodies および Alexa Fluor®488 Labeled Donkey Anti-Rat IgG Antibodies®を Can Get Signal®で 200 倍に希釈して室温で 60 分間反応させ、DAPI 1 μ g/ml 3 分間反応させた。PBS で洗浄後に封入した。

【結果】負荷を解除し 3 日経過した組織像は、牽引側で著明に細胞が増加

していた。1 週間経過したものでも、紡錘形の細胞が目立つ対照群と比べ、円形の細胞が新たに出現し、圧迫側と牽引側ともに確認できた。圧迫側では、対照群に比べ、継続して細胞数が増加していた。牽引側では、対照群との比較では大きく増加していた。GFP 骨髄移植マウスを用いた実験系では、経時的に GFP 陽性細胞数は、負荷直後から 6 か月まで緩やかに増加していた。免疫染色を行った GFP マウスにおける GFP 陽性骨髄由来の細胞は、円形、紡錘形など多様な形態を呈していた。本来、GFP 陽性細胞は、未分化間葉系の細胞であるため、形態は円形をしているが、今回の実験では紡錘形、多角形の細胞もあった。また、血管腔を形成するように染色された細胞があった。これらの GFP 陽性細胞は、CD31、CD68、Runx2 などとの蛍光二重染色により、マクロファージ、破骨細胞、血管内皮細胞、歯根膜線維芽細胞等に分化していることが明らかになった。

【考察】今回の実験で、歯科矯正学的メカニカルストレスは、圧迫側、牽引側ともに歯周組織における細胞数の増加を促すことが示唆された。細胞増加が短期間で起きている点から、細胞が歯周組織で増殖し、構成細胞へ分化しているとは考えにくい。GFP 陽性細胞は、ストレス負荷直後では緩やかな発現であったが、数週間で上昇し、6 か月には著明に増加したことから、骨髄由来細胞が長期間にわたり歯周組織へ供給されたと考えられる。蛍光免疫二重染色は、CD31 陽性細胞の一部は GFP 陽性を呈し、骨髄由来細胞か血管内皮細胞に移動、分化したことが明らかになった。さらに、CD68 の結果も同様であった。歯根膜線維芽細胞に特異的に発現している Runx2 に着目した実験では、GFP 陽性細胞と Runx2 を重ね合わせた二重陽性細胞が認められ、その形態が長紡錘形をしていることから、GFP 陽性の骨髄由来の歯根膜線維芽細胞だと判明した。

Research Paper

Histological Evaluation of Periodontal Ligament in Response to Orthodontic Mechanical Stress in Mice

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Abstract

The purpose of the study was to determine the cell dynamics in periodontal ligament in response to mechanical stress during orthodontic movement. Following Waldo's method, a square sheet of rubber dam was inserted in between the first and second maxillary molars in 10 ddY mice leaving the stress load for 3 hours. After 3 days and at 1 week, cell count on pressure and tension sides of the periodontal ligament was determined. Furthermore, the type of cell present after mechanical stress was identified using GFP bone marrow transplantation mouse model. Immunohistochemistry was carried out at 0 min (immediately after mechanical stress), 24 hours, 1 week, 2 weeks and 6 months. Temporal changes in the expression of GFP-positive bone marrow derived cells were examined. Moreover, double immunofluorescent staining was performed to determine the type of cell in the periodontal ligament. Cell count on the tension side tremendously increased 3 days after mechanical stress. At 1 week, spindle and round cell count increased compared to the control group. These changes were observed on both tension and pressure sides. Cell count on pressure side at 3 days (22.11±13.98) and at 1 week (33.23±11.39) was higher compared to the control group (15.26±8.29). On the tension side, there was a significantly increased at 3 days (35.46±11.85), but decreased at 1 week (29.23±13.89) although it is still higher compared to the control group (AD±SD: 10.37±8.69). Using GFP bone marrow transplantation mouse model, GFP positive cell count increased gradually over time in 6 months. GFP positive cells were also positive to CD31, CD68 and Runx2 suggesting that fibroblasts differentiated into osteoclasts and tissue macrophages. In conclusion, mechanical stress during orthodontic movement promoted the increase in the number of cells in the periodontal ligament on both tension and pressure sides. The increase in the number of cells in the periodontal ligament is believed to be due to the migration and cell division of undifferentiated mesenchymal cells.

Key words: Periodontal Ligament, Orthodontic Mechanical Stress, Cell movement, Cell differentiation, Immunohistochemistry

Introduction

It is well known that orthodontic appliance causes mechanical stress on the periodontal tissues leading to teeth movement. Aside from bone deposition by osteoblasts and bone resorption by osteoclasts, various studies have been done on periodontal tissue remodeling during orthodontic teeth movement.

However, studies on the origin of the fibroblast as well as the cell count in response to orthodontic teeth movement are only very few. Tomida et al. (1) focused on the pluripotency of bone marrow derived cells (BMDCs), since studies have mentioned their migration to the periodontal ligament (PDL) after receiving

mechanical stress. In addition, Muraoka et al. (2) revealed that BMDCs migrate into the PDL and differentiate into macrophages, osteoclasts, etc.

In the present study, we focused on the histopathological evaluation of cells in periodontal tissue remodeling caused by mechanical stress during orthodontic teeth movement. Furthermore, the source of cells was identified using GFP bone marrow transplantation mouse model by examining GFP-positive cell count immediately after mechanical stress until 6 months. Immunofluorescent double staining was carried out to identify the type of cells, which BMDCs have differentiated.

Materials and Methods

Laboratory animals

A total of ten, 8-week old male mice were purchased from SLC Japan Inc. (Hamamatsu, Japan). The mice were housed in metal cage lined with beddings (Paper clean: Peparlet Co., Ltd, Hamamatsu, Japan) in an air-conditioned controlled environment at 24 ± 1 °C. During breeding, the animals had free access to water and food (Picolab Rodent Diet 20: SLC Japan Inc.).

BMDCs were harvested from ten GFP transgenic mice and were transplanted into 7-week old C57BL/6 mice (3, 4). GFP transgenic mice were sacrificed by ether anesthesia followed by excision of femur, removal of soft tissue and harvest of bone marrow cells. The harvested bone marrow cells were washed with RPMI1640 medium containing antibiotics and then replaced with HBBS. On the other hand, GFP transgenic mice and their 7 week-old cognates were subjected to 10 Gray X-ray irradiation. Seven pieces of 1×10 squares of bone marrow cells were injected into the tail vein of recipient mice (5, 6). After 4 weeks of bone marrow transplantation, histological analysis of various tissues taken from the mouse was carried (Table 1).

Table 1. Periods and Number of Experimental Animals (ddY and GFP)

ddY	Experimental					Total
Periods	3 days		1 week			
Number	5		5			10
GFP	Experimental					Total
Periods	0 min	24 hours	1 week	2 weeks	6 months	
Number	2	2	2	2	2	10

Experimental methods

The animals were subjected to 4.0 % Isoflurane inhalation anesthesia (Isoflu: Animal Science Department, Dainippon Sumitomo Pharma Co., Ltd. Osaka, Japan), which is a mixture of gas and air. The

gas anesthesia system (DS Pharma Biomedical Co., Ltd. Laboratory Products, Osaka, Japan) used is for small laboratory animals with adjustable gas concentration and controlled flow rate in order to place the animals under stable condition. After adequate anesthesia was obtained, the mouse was fixed on a handmade bench in a sitting position and the nose attached to a suction hole to maintain general anesthesia. Isoflurane inhalation anesthesia was set to maintain the general anesthesia at a concentration of 1.0 % throughout the experiment. To keep the mouth open during experiment, the maxilla was fixed by tying the upper incisors to the upper part of the bench and the mandible was fixed by applying rubber on the lower incisors to the lower part of the bench. Following Waldo's method, the separator was inserted to induce mechanical stress on the PDL of maxillary molar. The separator used was a rubber dam sheet (Ivory, Premium Rubber Dam Pure Latex: Heraeus Kulzer, GmbH & Co. KG, Hanau, Germany), cut into approximately 2×2 square and folded into two. The mouse has 3 maxillary molars on each side numbered as maxillary first molar (M1), second molar (M2), third molar (M3). M1 and M2 have 3 roots and M3 has 2 to 3 roots. The separator was inserted between M1 and M2 and left for 3 hours in order to induce mechanical stress and compression of the PDL. When mechanical load was released, the maxilla with the molar and PL tissue were excised 3 days and 1 week later.

In GFP bone marrow transplanted mice, mechanical load was applied for 3 hours in the same manner as above. After 24 hours, 1 week, 2 weeks and 6 months, the portion of the maxillary periodontal tissue was excised from the maxilla.

For the control or non-treated group, the opposite side of the maxillary molar PDL was used. The distal side of the buccal roots of M1 was used as the observation site. The experiment was carried out following the animal experimental guidelines of the ethics committee of Matsumoto Dental University.

Tissue preparation

After excision, the tissues were placed in 4 % paraformaldehyde with 0.05 M phosphate buffer fixing solution for 24 hours and then demineralized in 10 % EDTA solution for 3 weeks. Then after, the specimens were embedded in paraffin; serial sections were made with a thickness of 5mm horizontally to the PDL, stained with HE and examined under the light microscope. To confirm the cell count in the PL on the pressure and tension sides, the entire tissue was carefully examined for cell morphology and areas with minimal changes were chosen. Then after, cell count in square pixels using Adobe Photoshop Ele-

ment 13 was carried out. Note that gaps such as lumen of the blood vessel were excluded.

Immunohistochemistry

For immunohistochemistry, specimens were deparaffinized in xylene and placed in an incubator at 60 °C for 30 min. Then after, the specimens were pre-treated in 0.03 % hydrogen peroxide methanol solution and then immersed in 10mM citric acid buffer with a pH of 6.0 and placed in autoclave at 121 °C for 5 min.

Anti-GFP ChIP grade ab290 (Abcam, Cambridge, UK) with a dilution of 1:5000 was allowed to react overnight at 4 °C. For the secondary antibody, anti-rabbit polyclonal antibody Simple Stain Mouse MAXPO® (Nichirei, Tokyo, Japan) was allowed to react, then washed with PBS followed by color development with DAB. In a similar manner, the GFP positive cells were counted under the microscope.

Immunofluorescence Double Staining

Anti-CD31, anti-CD68 and Runx2 antibodies were combined with anti-GFP rabbit polyclonal anti-

body. After deparaffinization, pre-treatment was done with citric acid buffer in microwave for 1 min. Blocking was carried out with 10 % donkey for 30 min, followed by reaction with primary antibody at 4 °C overnight. For secondary antibody, Alexa Fluor R568 Donkey, anti-goat IgG antibodies (Molecular Probes, Japan) and Can Get Signal (Toyobo, Japan) with dilution of 1:200 was incubated for 60 min. Staining of the nucleus with 1 mg/ml DAPI was carried out for 3 min. Finally, slides were washed with TBS and then mounted using Fluorescent Mounting Medium (DAKO).

Results

Histopathological findings (ddY mice group: Fig. 1)

In the control group, the PDL runs radially from the cementum and alveolar bone and the bundles are relatively arranged in order. The spindle shaped fibroblasts have oval nucleus running parallel to the PL fibers.

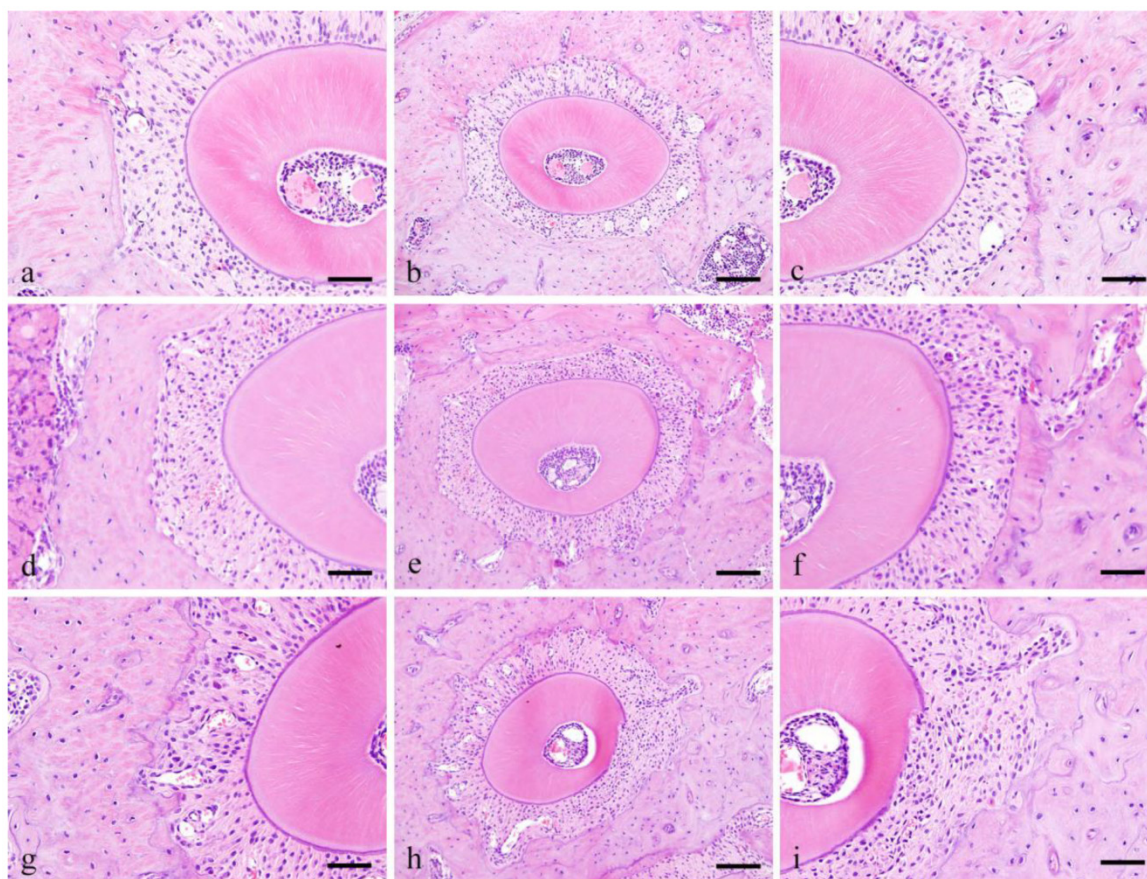


Figure 1. Histopathological views of control specimens. Center column shows whole alveolar bone socket morphology (b, e, f; Scale bar=200µm). Left column is high power-view of PDL tension side (a, b, c; Scale bar=100µm), and right is pressure side (c, f, i; Scale bar=100µm).

In the Experimental group, at pressure side, 3 days, the PDL space became narrow and densely packed with cells that are irregularly arranged. The size of nucleus varies and the shape is round compared to the spindle shape cells in the control group. Pressure side, 1 week, cell count at 1 week is higher compared to 3 days. Cells were both localized in alveolar bone and cementum and several osteoclasts were observed adjacent to the alveolar bone. The nuclei are huge and occupy most of the cytoplasm and are more round in shape compared to the control group.

Tension side, 3 days, cell count at 3 days is higher compared to the control group. The cells are somehow spindle in shape with round shaped cells in between. Tension side, 1 week, cell count is slight higher compared to 3 days but clearly higher compared to control group. The cells present are both round and spindle in shape.

Cell counting

Cell count on pressure and tension sides were calculated by counting the number of nuclei in square pixels in selected areas. In pressure side, compared to the control group (15.26 ± 8.29), a gradual increase in cell count was obtained at 3 days (22.11 ± 13.98) and continued further at 1 week (33.23 ± 11.39) (Fig. 2).

In tension side, compared to the control group ($AD \pm SD$: 10.37 ± 8.69), cell count significantly increased at 3 days (35.46 ± 11.85) but decreased at 1 week (29.23 ± 13.89) although it is still higher than the control group. The significant difference was confirmed by Scheffe test (Fig. 3).

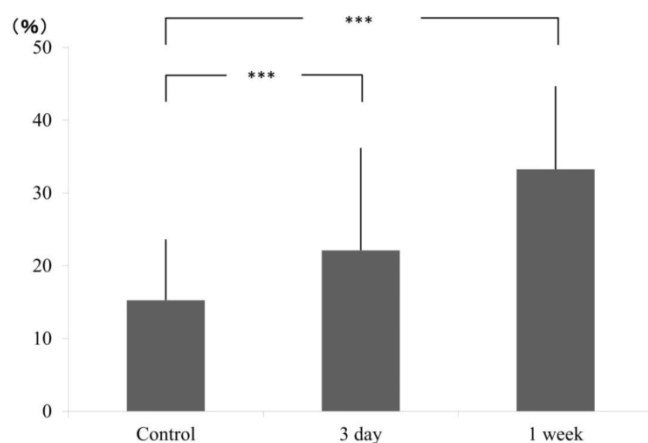


Figure 2. Ratio of pixel number in the control and experimental (3 day and 1 week), pressure side.

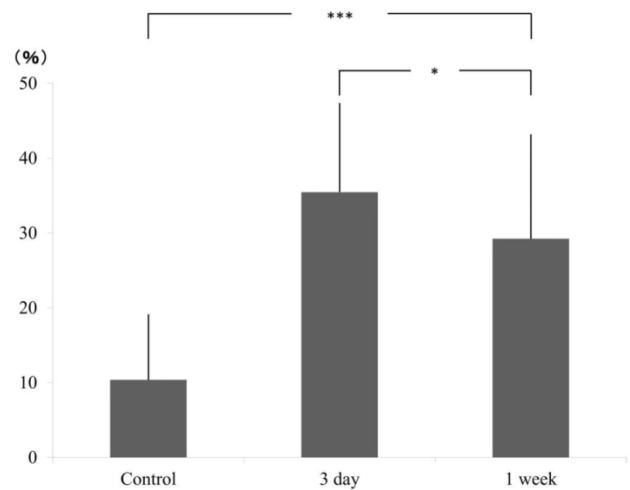


Figure 3. ratio of pixel number in the control and experimental (3 day and 1 week), tension side.

GFP mouse examination

Histopathological findings

In the control group, cells on both pressure and tension sides are few. Spindle cells were observed on the pressure side while round cells were mostly observed on the tension side. In 0 min (immediately after mechanical stress loading) specimens, cell count increased on both tension and pressure sides. On the pressure side, the cells are round in shape while on the tension side, the cells are spindle in shape.

In 24 hours, cell count increased on both tension and pressure sides. Spindle shape cells are observed on both sides. In 1 week-specimens, there was no change in cell count on both tension and pressure sides compared to 24 hours. The cells are small and large with round or spindle shape nuclei. In 2 weeks, there was no change in the cell count on both pressure and tension sides but cells are more round in shape on the pressure side. In 6 months specimens, cell count is similar to the control group but round cells are more prominent on the pressure side.

GFP-positive-cell counting

Immediately after mechanical stress (0 min), some GFP positive cells were observed on the pressure side. At 24 hours, GFP-positive cells slightly increased and the cells are round in shape. At 1 week, GFP-positive cells increased on both pressure and tension sides. At 2 weeks, GFP-positive cells further increased compared to 1 week. At 6 months, GFP-positive cell was predominantly observed on the tension side.

Immunohistochemical findings

BMDCs are positive to GFP except for those at peripheral capillaries. Most GFP positive cells are

located in alveolar bone near the PL and exhibit various shapes such as round, spindle, etc. Certainly, GFP-positive cells have the morphology of undifferentiated mesenchymal cells (UMC), which are fusiform, round or even polygonal. In addition, cells lining the lumen of the blood vessel are positive to GFP (Fig. 4a-b).

GFP and immunofluorescent double staining examination deals the specimens were subjected to double staining to identify various cell types of GFP-positive cells. Immunostaining of GFP-CD31, GFP-CD68 and GFP-Runx2 are shown in Fig. 5a-d respectively. Fig. 5 shows GFP positive cells in green fluorescence, Runx2 positive cells in red fluorescence and superimposition in orange fluorescence. Fusiform cells are arranged in bundles towards the root of the tooth. When superimposed with DAB, the nuclei stained blue and the periphery were stained with orange.

Discussion

Recently, bone marrow derived un-differentiate mesenchymal cells (UDMC) has been widely used in

studies on repair and regeneration of periodontal tissue, bone and other organs. During the regeneration of alveolar bone and PDL, studies have mentioned the differentiation of UDMC from bone marrow into cartilage (7). Experiments on GFP mouse and BMDCs showed that after acinar cell transplantation, the cells differentiated into salivary gland components such as ductal cells and myoepithelial cells with the ability to play a role in salivary gland tissue (8), as well as into olfactory cells (9). Histological experiment on rats subjected to orthodontic load showed that bone marrow stem cells increased the bone remodeling capacity (10).

Tomida et al. (1) measured the increase in PDL cells after mechanical stress at 1 and 5 weeks. In the present study, cell count was determined by counting GFP positive cells in the control and experimental groups. Results showed that GFP positive cell count in the experimental group is significantly higher than the control group. The increase in cell count in the PDL after mechanical stress highly suggests that the transplanted BMDCs were activated to migrate towards the PDL.

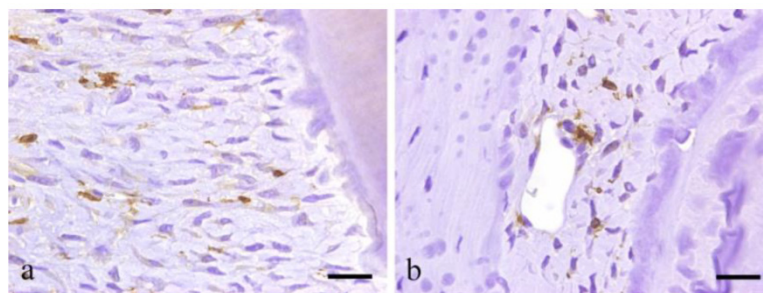


Figure 4. Immunohistochemical results showing GFP-positive round- or spindle-shaped cells within the PDL tissues (a; scale bar=50 μ m), and GFP-positive products existing within the vascular endothelial cells (b; scale bar=50 μ m).

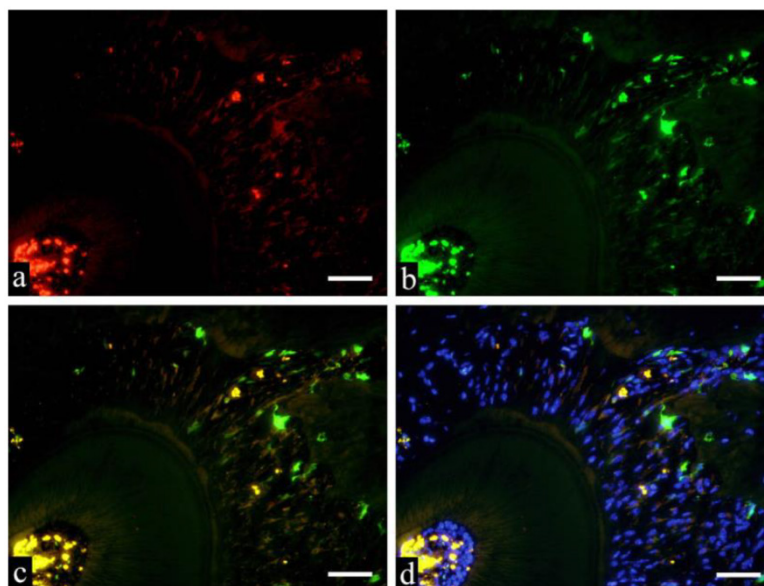


Figure 5. Immunofluorescent staining images of PDL (a: Runx2; b: GFP; c: merged image of Runx2 and GFP; and d: merged image of Runx2, GFP and DAPI; 6 months specimen; scale bar= 100 μ m).

Tsujigiwa et al. (11), using GFP mouse experiments showed that transplanted BMDCs migrated into the pulp and differentiated into cells that constitute the pulp tissue. Using the same experiment, Muraoka et al. (2) showed that transplanted BMDCs migrated into PDL and then later differentiated into PDL cells. However, the biological reaction of mesenchymal cells in response to mechanical stimulus also led to the change in the cell morphology (12). The migration of transplanted BMDCs using GFP mouse was reported to be due to mechanical stimulation (9).

The results of the study suggested that mechanical stress promoted the increase in cell count in PL on both pressure and tension sides. Cell count increased in as short as 1 week although change in cell morphology was not detected. To determine if the increase in cell count was due to the migration of BMDCs, GFP positive cells were counted in the same manner done by Tomida et al. (1). A gradual increase in GFP positive cell count immediately after stress was noted, which further increased over time until 6 months. It is believed that PL was supplied with BMDCs for a long period of time. Furthermore, immunofluorescent double staining was performed to characterize the cells that have differentiated. Tsujigiwa et al. (13) confirmed that transplanted BMDCs migrate to the area of bone repair shown by double staining with GFP and CD34 in osteoclasts, etc. Applying the methodology of Muraoka et al. (2), we were able to identify the differentiation of cells into osteoclasts and macrophages by double immunofluorescent staining with GFP, CD31, CD68 and Runx2. Results showed that some GFP-positive cells that also expressed CD31 are either BMDCs or vascular endothelial cells and their differentiation was evident. Similar results were obtained with CD68. Moreover, the expression of Runx2 was carried out since it is also expressed by fibroblast (14-16). Results showed that the cells that co-expressed GFP and Runx2 are long and spindle shaped PL fibroblasts strongly suggesting that the cells were derived from bone marrow.

Based on the results and discussion, the following conclusions have been obtained:

1. Orthodontic mechanical stress promotes the growth of cells in the PDL.
2. Using GFP mouse, cell proliferation was brought about by the migration of BMDCs into the PDL.
3. BMDCs that migrated to the PDL differentiated into vascular endothelial cells, macrophages, osteoclasts and fibroblasts.

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Competing Interests

The authors have declared that no competing interest exists.

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