Effect of local bone marrow stromal cell administration on ligature-induced periodontitis in mice

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Abstract: Bone marrow-derived multipotent stromal cells (BMSCs) have potent anti-inflammatorv effects. This study aimed to investigate the anti-inflammatory potential of BMSCs using a mouse model of ligature-induced periodontitis. BMSCs were isolated from the femurs and tibias of mice. Periodontitis was induced by placing a ligature around the right maxillary second molar. After 3 days, the mice were administered BMSC in the gingiva of the mesial interdental papilla around the ligatured molar. The ligatured and non-ligatured mice that were not administered BMSC served as controls. Differences in inflammatory infiltration and bone resorption around the roots of the second molar were assessed and were subsequently quantified using microcomputed tomography (micro-CT), histological analysis, and tartrate-resistant acid phosphatase (TRAP) staining. Micro-CT revealed that alveolar bone loss around the ligatured molars increased in a time-dependent manner; however, the effect was significantly less in BMSC-treated mice compared with ligatured control mice. Tissue histopathology revealed that BMSC administration mitigated inflammatory infiltration in ligatured BMSC mice. In addition, the number of TRAP-positive osteoclasts was markedly elevated in ligatured control mice compared with those in BMSC-treated mice. These findings indicate that local BMSC administration can mitigate inflammation and alveolar bone resorption, suggesting that administering BMSC leads to new therapeutics for periodontitis.

Keywords: bone marrow stromal cell; bone resorption; cell therapy; local administration; periodontitis.
Introduction

Periodontitis is a common chronic dental disease worldwide, and based on a national survey, it is present in 42.5% of Japanese citizens (1,2). The onset of this disease results from bacterial plaque accumulation that elicits an inflammatory cascade, leading to leukocyte infiltration, connective tissue destruction, pocket formation, and alveolar bone resorption (3-5). Although the initial inflammatory reaction is generally reversible as represented by gingivitis, an untreated disease can progress to a chronic state that is characterized by inflammation that extends from the gingival tissue to the adjacent alveolar bone and periodontal ligament. Because these periodontal structures play a critical role in supporting teeth, failure to treat periodontitis can result in tooth loss. The primary goal of currently applied therapeutics for periodontitis is reducing the subgingival bacterial load via instrumental debridement with surgical or non-surgical approaches (6,7). However, entire removal of the resulting biofilm when bacterially affected root surfaces are embedded in soft tissues or located in anatomically inaccessible areas is almost impossible; antibiotics or antiseptics are often administered as adjuvant treatments (8). Therefore, some periodontitis cases can be unresponsive to a conventional therapy, calling for the development of novel therapeutic regimens for periodontitis.

Multipotent mesenchymal stromal cells (MSCs) are postnatal stem cells that are found in various human tissues. They possess self-renewing ability and can differentiate into several cell lineages, including osteocytes, chondrocytes, and adipocytes (9-12). Most studies on MSCs have focused on their regenerative properties; however, recent studies have demonstrated the profound antiinflammatory effect of MSCs by secreting factors in response to activation cues from injured tissues (13,14). In brief, the cells can migrate to sites of injury and promote cell survival and proliferation via direct cell-to-cell contact or paracrine activity. These actions, combined with their accessibility and in vitro expansion potential, make MSCs an attractive candidate for antiinflammatory treatments. As such, this study investigated the possible therapeutic effects of Bone marrow-derived multipotent stromal cells (BMSCs) in an experimental animal model of progression stage of periodontitis.

Materials and Methods

Animals

All animal experiments were performed according to the guidelines of The Animal Research and Care Committee at the Nihon University School of Dentistry. The Animal Experimentation Committee of Nihon University School of Dentistry approved the study protocols (AP15D027). Eight-week-old male wild-type C57BL/6 mice (CLEA Japan, Inc., Tokyo, Japan) were housed in an experimental animal room (22°C, 55% humidity, 12-h light/dark cycle). The mice were divided into the following three groups (n = 45 total; 15 per group): 1) ligature + BMSCs, 2) ligature control (ligature + PBS vehicle administration), and 3) no ligation control.

Isolation and culture of BMSCs

BMSCs were isolated from the femurs and tibiae of 8-week-old C57BL/6 mice, as previously described (15). In brief, the femurs and tibiae were washed thrice in fresh PBS with vigorous shaking and then crushed with a sterile pestle and mortar, and the fragments were flushed thrice with 10 mL Hanks balanced salt solution. The flushed bone fragments were then transferred to a 50-mL conical tube and incubated with 20 mL preheated Dulbecco’s modified Eagle’s medium (DMEM) (Sigma Aldrich, St. Louis, MO, USA) supplemented with 0.2% (wt/vol) collagenase (Wako Pure Chemical Industries, Osaka, Japan) for 1 h. The resulting cell suspension was filtered through a 70-μm cell strainer and then placed on ice for 70 min to quench collagenase activity. After centrifugation, the pellet was treated with ice-cold sterile H₂O for 6 s to lyse red blood cells, leaving only BMSCs in the resulting pellet. Cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% antibiotics at 37°C in a humidified atmosphere that contained 5% CO₂, with daily medium changes.

Cell surface marker analysis

The phenotype of BMSCs was confirmed using flow cytometry for surface marker stem cell antigen-1 (Sca-1). Cells were harvested with trypsin and incubated with the Sca-1-FITC antibody (BD Biosciences, San Jose, CA, USA) for 30 min (16); they were then stained with 1 μg/mL propidium iodide (Sigma Aldrich). Flow cytometry data were analyzed using the FlowJo software (Treestar, San Carlos, CA, USA).

Induction of progression stage of periodontitis

Eight-week-old male mice were intraperitoneally anesthetized using a mixture of medetomidine (0.15 mg/kg), midazolam (2 mg/kg), and butorphanol tartrate (2.5 mg/kg). To induce periodontitis, a 5-0 silk ligature was tied around the right maxillary second molar and sutured to prevent periodontal tissue damage (17). Ligatures were checked daily and maintained in place for all mice.
throughout the 14-day experimental period (n = 30). Body weights were recorded daily throughout the experimental period; no differences between the ligatured and non-ligatured control groups were observed.

Determination of cell dosage and administration in mice
We determined the optimal cell dose to prevent bone loss in this mouse model. In brief, three BMSC concentrations (10^4, 10^5, or 10^6 cells in 5 µL PBS) were administered into the gingiva of the mesial interdental papilla at the second molar using a 33-G syringe (Hamilton, Reno, NV, USA) at 3 days after ligature placement (n = 30 total; 15 per group). The bone loss was then monitored using microcomputed tomography (micro-CT) and histological analysis as described below.

Micro-CT imaging and analysis
The RmCT system (Rigaku Co., Tokyo, Japan) has a microfocus X-ray tube with a 7-µm focal point and a sensor with a 4-inch image intensifier. The X-ray source and image intensifier were connected by a basal plate, and the I-arm rotated on the vertical plane and was driven by a direct-drive motor. The mice were placed on the stage to image the regions of interest with exposure parameters at 90 kV and 100 µA (18-20). The size of the regions of interest was 1 × 1 × 3 mm. The bone volume (BV) in the regions of interest was measured to assess bone resorption. Images were reconstructed using the i-View software. Cortical BV in the regions of interest was measured using the micro-CT imaging data in the BV measurement software (Kitasenju Radist Dental Clinic I-View Image Center., Tokyo, Japan). For this, gray values and the corresponding number of voxels were calculated and compared with that from the first day of imaging at days 3, 5, 7, and 14 post-ligature placement.

Tissue histology
Inflammation and alveolar bone loss were assessed by hematoxylin and eosin staining. In brief, the tissue was harvested from euthanized animals and fixed in 10% neutral buffered paraformaldehyde for 48 h, decalcified in 15% EDTA for 5 weeks, dehydrated using a graded ethanol series, and then embedded in paraffin. Specimens (n = 45 total) were sagittally sectioned (4-µm thick) with a microtome (Leica RM2165, Nussloch, Germany) and then stained with hematoxylin and eosin. For the quantitative analysis, five stained sections from each specimen were selected, and alveolar bone loss was quantified by measuring the distance between the cementoenamel junction (CEJ) and alveolar bone crest (ABC) in the mesial and distal roots of the maxillary second molar and between the roof and ABC in the furcation area using light microscopy (Eclipse LV100POL and Digital Sight DS-L2; Nikon, Tokyo, Japan). Gingival recession was also determined by measuring the distance between CEJ and the apical termination of the junctional epithelium.

Tartrate-resistant acid phosphatase staining
Osteoclasts and odontoclasts were defined as multinucleated (≥3) tartrate-resistant acid phosphatase (TRAP)-positive cells in contact with the surface of the alveolar bone and root cementum of furcation sections, respectively. At 4 and 11 days after BMSC administration (7 and 14 days post-ligature placement), osteoclasts and odontoclasts were detected by TRAP staining performed using a leukocyte acid phosphatase kit (Sigma Adrich) (21). Images were captured using a microscope (Eclipse LV100POL; Nikon, Tokyo, Japan), and TRAP-positive cells were counted using the Image J software (NIH, Bethesda, MD, USA).

Localization of DiI-labeled BMSCs
BMSCs (wild-type C57BL/6) were harvested with 0.25% trypsin-EDTA, resuspended at 10^6 cells/mL in DMEM, and then labeled with the fluorescent tracer dye DiI (5 µL/mL, Molecular Probes, Eugene, OR, USA) for 20 min. Stained cells were centrifuged at 280 g for 5 min and washed twice with PBS before administration into recipient mice. The survival and localization of BMSCs were then examined at 0 and 4 days after cell administration (19).

Statistical analysis
Statistical comparisons between the groups were performed using one-way analysis of variance, followed by Tukey-Kramer post hoc test. Statistical analysis was conducted using GraphPad Prism software version 6 (San Diego, CA, USA). Statistical significance was defined as P < 0.05.

Results
In vitro characterization of multipotent BMSCs
BMSCs isolated from C57/B6 mice were capable of expanding in vitro and formed fibroblast-like colonies after culturing for 7 days in a MSC growth medium (Fig. 1A). Flow cytometry showed that 40% of cells expressed the mesenchymal stem cell marker Sca-1 (Fig. 1B), consistent with a previous study (22). Therefore, the cultured cell populations were positive for key stem cell markers such as Sca-1.
Ligature-induced periodontitis and BMSC efficacy

Preliminary analyses were performed to determine the optimal cell number of BMSC administration (10^4, 10^5, or 10^6), including Sca-1-positive and Sca-1-negative cells for therapeutic efficacy. Bone loss was estimated using micro-CT and histological images in each cell number group at 7 days after ligature placement (Fig. 2A). Quantification analysis demonstrated that the mice administered 10^6 BMSCs retained significantly more BV compared with those administered 10^4 or 10^5 BMSCs (Fig. 2B). Furthermore, based on the histological evaluation at day 7 after administration, a remarkable infiltration of mononuclear cells was observed in the furcation area of mice administered 10^4 or 10^5 cells, but not 10^6 cells (Fig. 2C). Because a significant favorable effect was observed in mice administered 10^6 BMSCs, we selected this dosage for our subsequent analyses.

Fig. 1 In vitro characterization of BMSCs. (A) Microscopic image of primary BMSCs harvested from C57/BL6 mice. (×100 magnification). (B) BMSC immunophenotyping by flow cytometry. Solid bars, Sca-1 antibody; open bars, IgG isotype controls; FITC, fluorescein isothiocyanate.

Fig. 2 Analysis of BMSC efficacy using micro-CT and histological imaging on day 7 after ligature placement (4 days after cell administration). (A) Two- and three-dimensional images in the sagittal and horizontal planes. (B) Alveolar bone loss in the furcation area of the maxillary second molar at the indicated time point after cell administration on days 3, 5, and 7 after ligation. (C) Low-magnification images of alveolar bone loss (a-c). High-magnification images of alveolar bone loss in furcation area (d-f). ab, alveolar bone; p, pulp; r, tooth root; M1, first molar root; M2, second molar root; M3, third molar root. a-c; scale bars, 1000 μm. d-f; scale bars, 100 μm. Data are expressed as mean ± standard deviation (SD). n = 5 per group, *P < 0.05, **P < 0.01 by one-way ANOVA test.

Fig. 3 Micro-CT analysis of alveolar bone loss after ligature placement and/or BMSC administration. (A) Micro-CT images from the same mice on days 3, 5, 7, and 14 after ligation (0, 2, 4, and 11 days after cell administration). CT showed 3D images of the sagittal and horizontal sections of the right maxilla periodontal tissue. (B) Quantification of hard tissue (bone) volume loss in all three groups. Data are expressed as mean ± SD. n = 5, **P < 0.01 by one-way ANOVA test.
Periodontitis-induced bone resorption

Micro-CT analysis showed no alveolar bone resorption in the no ligation control group at 14 days after placement, as expected (Fig. 3A). However, 50% of the alveolar bone in the mesial and distal roots was resorbed in the ligature control group at 5 days after ligature placement, whereas only 33% was resorbed in the ligature + BMSC group (Fig. 3B). Horizontal section analysis revealed that the buccal and distal root alveolar bones were completely resorbed at day 7 and 14, respectively, in the ligature control group. In comparison, the mesial root was still embedded within the alveolar bone in the ligature + BMSC group at day 14 (Fig. 3A). Moreover, BMSC-treated mice had markedly less bone loss than the ligature control mice after 5 days (Fig. 3B; P < 0.01).

Histological analysis

Representative low-magnification histological images revealed a gradual decrease in alveolar bone height and complete resorption on day 14 in the ligature control group (Fig. 4A, B). Analysis of inflammatory cell infil-
trate also showed a marked decrease in mononuclear cells in the medial and distal lamina propria under the gingival epithelium in the ligature + BMSC group compared with that in the ligature control group on day 5 (Fig. 4C). Moreover, principal gingival and periodontal ligament fibers were clearly observed in the mesial area of BMSC-treated mice but not in the ligature control mice (Fig. 4C). In addition, BMSC-untreated ligatured mice exhibited a marked increase in polymorphonuclear cells in the alveolar bone and root cementum surface within the furcation area, which were subsequently identified as osteoclasts and odontoclasts by TRAP staining (Fig. 5).

Table 1 CEJ-ABC or furcation-ABC distances in the distal, mesial, and furcation areas

| Site          | No ligation (µm) | Ligature (µm) | Ligature + BMSC (µm) | No ligation (µm) | Ligature (µm) | Ligature + BMSC (µm) |
|---------------|------------------|---------------|----------------------|------------------|---------------|----------------------|
| Distal        | 0.52 ± 0.11      | 5.90 ± 0.69   | 2.10 ± 0.39*         | 0.56 ± 0.10      | 6.10 ± 0.38   | 3.10 ± 0.45*         |
| Furcation     | 0.42 ± 0.25      | 5.55 ± 0.42   | 1.90 ± 0.29*         | 0.46 ± 0.42      | 5.83 ± 0.50   | 2.93 ± 0.42*         |
| Mesial        | 0.59 ± 0.15      | 4.98 ± 0.55   | 2.03 ± 0.48*         | 0.62 ± 0.12      | 5.75 ± 0.75   | 2.22 ± 0.35*         |

*P < 0.05 compared with the ligature group by one-way ANOVA test (n = 5).

Table 2 Gingival recession distance in the distal and mesial roots

| Site          | No ligation (µm) | Ligature (µm) | Ligature + BMSC (µm) | No ligation (µm) | Ligature (µm) | Ligature + BMSC (µm) |
|---------------|------------------|---------------|----------------------|------------------|---------------|----------------------|
| Distal        | 0.00 ± 0.00      | 2.75 ± 0.69   | 1.35 ± 0.37*         | 0.00 ± 0.00      | 3.25 ± 0.55   | 1.72 ± 0.82*         |
| Mesial        | 0.00 ± 0.00      | 2.62 ± 0.35   | 1.32 ± 0.40*         | 0.00 ± 0.00      | 3.35 ± 0.40   | 1.60 ± 0.15*         |

*P < 0.05 compared with the ligature group by one-way ANOVA test (n = 5).

Quantification of alveolar bone loss

BMSCs from untreated, ligatured teeth showed significant increases in the CEJ-ABC and furcation-ABC distances throughout the experimental period consistently in our histological analysis. The ligature control group showed markedly greater CEJ-ABC distances than the ligature + BMSC group at 7 and 14 days after ligature placement (Table 1 and Fig. 6; double-headed arrows; P < 0.05). In addition, BMSC treatment significantly reduced gingival recession as determined by the distance between CEJ-the apical termination of the junctional epithelium (Table 2; Fig. 6B; double-headed arrows; P < 0.05).
To determine the localization of BMSCs after administration, BMSCs labeled with DiI were administered in the gingiva of the mesial interdental papilla at the mesial root in the second molar of recipient mice. At 1 h and 4 days after BMSC administration, the tissue sections were harvested and examined for DiI emission. Notably, BMSCs were observed within the gingival epithelium at 1 h after administration, whereas BMSCs also appeared in the lamina propria of gingiva after 4 days (Fig. 7).

**DiI-labeled BMSC tracking**

To determine the localization of BMSCs after administration, BMSCs labeled with DiI were administered in the gingiva of the mesial interdental papilla at the mesial root in the second molar of recipient mice. At 1 h and 4 days after BMSC administration, the tissue sections were harvested and examined for DiI emission. Notably, BMSCs were observed within the gingival epithelium at 1 h after administration, whereas BMSCs also appeared in the lamina propria of gingiva after 4 days (Fig. 7).

**Discussion**

This study aimed to investigate the effect of locally administered BMSCs, including Sca-1-positive cells, on periodontal tissue destruction in a mouse model of progression stage of periodontitis. Various animal models have been developed to understand the etiology of periodontal diseases and characterize potential therapies (23,24). Ligature-induced periodontitis is one of the most widely used evidence-based disease models (25). Our model clearly showed increased alveolar bone resorption and inflammatory cell infiltration, indicating that the findings were consistent with those from a previously reported mouse model of ligature-induced periodontitis (19).

BMSCs can be administered by several methods such as systemic intravenous injection or local injection into the tissue of interest. Of these, intravenous injection is the most widely used because of the following advantages: 1) intravenous injection is minimally invasive, 2) BMSC infusions are reproducible, and 3) administered cells remain close to the oxygen- and nutrient-rich vasculature of the target tissue (27). However, many infused BMSCs may be trapped by some organs after intravenous injection, interrupting the homing of BMSCs to the target tissues. Direct BMSC administration is not the preferred for kidney and liver diseases. Conversely, local injection is clinically feasible for treating periodontitis because the injections can be directly administered into the gingiva. A previous study by our group demonstrated that cells implanted in artificial periodontal defects resulted in the regeneration of periodontium within 2 weeks from cell implantation (19). Furthermore, some studies suggest that MSCs have the ability to engraft damaged tissues, integrate into tubular cells, and differentiate into mesangial cells (28,29). Nevertheless, directly administered BMSCs may exhibit diminished survival by the lack of available oxygen or nutrients in some target tissues (27). In this study, the locally administered DiI-labeled BMSCs were detected at 4 days after cell administration in ligature-induced progression of periodontitis in conjunction with the diminished periodontal bone resorption level, indicating that this method was potent in preventing periodontitis progression. However, further studies are necessary to determine the survival rate of administered BMSCs over a time span of >4 days.

Next, we examined the effect of BMSCs dosage on the extent of treatment response. We found that the administration of 10⁶ cells was significantly better at suppressing alveolar bone loss than other doses examined. Therefore, this dosage was selected for subsequent analyses.

BMSCs were administered 3 days after ligature placement with a single dose into the mesial area of the root because previous studies showed that inflammation arises within 2 days of ligature placement, beginning around the cervical area and eventually leading to a chronic inflammatory response that results in alveolar bone loss (30-32). The ligature was maintained in the subgingival sulcus for the entire experimental period because its removal facilitated periodontal repair (33).

The location of BMSC administration was carefully considered. We administered BMSCs into the mesio-buccal gingiva at the second molar root; however, a greater difference was observed in alveolar bone loss and periodontal integrity between the mesial and distal areas at the second molar root in the ligature + BMSC group, suggesting that the cell microenvironment regulates the effect of locally administered BMSCs. However, further analysis is necessary to completely understand these effects.

Our analysis of alveolar BV revealed a significant decrease in the ligature control group vs. the ligature + BMSC group. In particular, the alveolar bone comprising the buccal wall of the second molar roots was clearly recognized in the ligature + BMSC group compared with that in the ligature control group. Moreover, fewer...
osteoclasts and odontoclasts were present in BMSC-administered mice, suggesting that these cells possibly ameliorated alveolar bone resorption by blocking osteoclast and odontoclast recruitment. Furthermore, BMSC-treated mice showed superior preservation of the principal fiber structure in the gingival and periodontal ligaments, indicating that BMSCs were well distributed over the connective attachment. Within the limitations of this study, the results showed that BMSC administration facilitated periodontal preservation in a mouse model of periodontitis because the administered cells could be effectively distributed in vivo.

Despite our findings, the mechanism by which BMSCs mitigate the effects of periodontitis remains unclear. One possibility is that BMSCs differentiate into cementoblasts, osteoblasts, and periodontal fibroblasts in vivo. However, in our study, Dil-labeled BMSCs were observed only in the gingiva, and no supporting evidence exists to suggest that BMSCs can differentiate into these cell types. A more likely possibility is that BMSCs suppress proinflammatory biological mediators because these cells exert profound antiinflammatory and immunomodulatory effects via various mechanisms that are most remarkable for cytokine and chemokine secretion (23,34-36). Nevertheless, further studies are warranted to evaluate the effects of immunomodulators on host inflammatory response.

MSCs can act through multiple mechanisms to coordinate a dynamic, integrated response to periodontitis, and additional studies to examine these multicellular effects are warranted. The attractive outcomes observed in this study, in addition to the known antiinflammatory functions of BMSCs, support their continued investigation as a potential treatment modality for periodontal diseases.

In conclusion, this study demonstrates that BMSCs can act through multiple mechanisms to coordinate a dynamic, integrated response to periodontitis, and additional studies to examine these multicellular effects are warranted. The attractive outcomes observed in this study, in addition to the known antiinflammatory functions of BMSCs, support their continued investigation as a potential treatment modality for periodontal diseases. Additional studies to examine these multicellular effects are warranted. The attractive outcomes observed in this study, in addition to the known antiinflammatory functions of BMSCs, support their continued investigation as a potential treatment modality for periodontal diseases.

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Conflict of interest
None declared.

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