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Full length article

Multipotent mesenchymal stromal cell sheet therapy for bisphosphonate-related osteonecrosis of the jaw in a rat model



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ABSTRACT

Bisphosphonates (BPs) inhibit bone resorption and are frequently used to treat osteoporosis, bone metastasis, and other conditions that result in bone fragility. However, numerous studies have reported that BPs are closely related to the development of osteonecrosis of the jaw (BRONJ), which is an intractable disease. Recent studies have demonstrated that intravenous infusion of multipotent mesenchymal stromal cells (MSCs) is effective for the treatment of BRONJ-like disease models. However, the stability of injected MSCs is relatively low. In this study, the protein level of vascular endothelial growth factor in BP-treated MSCs was significantly lower than untreated-MSCs. The mRNA expression levels of receptor activator of nuclear factor κ -B ligand and osteoprotegerin were significantly decreased in BP-treated MSCs. We developed a tissue-engineered cell sheet of allogeneic enhanced green fluorescent protein (EGFP)-labeled MSCs and investigated the effect of MSC sheet transplantation in a BRONJ-like rat model. The MSC sheet group showed wound healing in most cases compared with the control group and MSC intravenous injection group (occurrence of bone exposure: 12.5% compared with 80% and 100%, respectively). Immunofluorescence staining revealed that EGFP-positive cells were localized around newly formed blood vessels in the transplanted sub-mucosa at 2 weeks after transplantation. Blood vessels were significantly observed in the MSC sheet group compared to in the control group and MSC intravenous injection group (106 ± 9.6 compared with 40 ± 5.3 and 62 ± 10.2 vessels/mm², respectively). These results suggest that allogeneic MSC sheet transplantation is a promising alternative approach for treating BRONJ.

Statement of Significance

Bisphosphonates are frequently used to treat osteoporosis, bone metastasis of various cancers, and other diseases. However, bisphosphonate related-osteonecrosis of the jaw (BRONJ) is an intractable disease because it often recurs after surgery or is exacerbated following conservative treatment. Therefore, an alternative approach for treating BRONJ is needed.

In this study, we developed a bone marrow-derived multipotent mesenchymal stromal cell (MSC) sheet to treat BRONJ and investigated the effect of MSC sheet transplantation in a rat model of BRONJ-like disease. The MSC sheet transplantation group showed wound healing in most cases, while only minimal healing was observed in the control group and MSC intravenous injection group. Our results suggest that the MSC sheet is a promising alternative approach for the treatment of BRONJ.

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1. Introduction

Bisphosphonates (BPs) inhibit bone resorption by inducing the apoptosis of osteoclasts [1]. They are frequently used to treat osteoporosis [2], skeletal-related events with bone metastasis [3], hypercalcemia, multiple myeloma, Paget's disease of bone, osteogenesis imperfecta, and other conditions that result in bone

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fragility [4]. However, after a report by Marx, an increasing number of reports have suggested that BPs are closely related to the development of osteonecrosis of the jaw [5–7]. BPs-related osteonecrosis of the jaw (BRONJ) is defined as exposed bone in the maxillofacial region that is resistant to conventional therapy for more than 8 weeks in patients taking BPs who have no history of radiation therapy to the jaws [4,8]. Previous studies and reviews reported that the risk of BRONJ in patients receiving intravenous BPs was approximately 1% [9,10] and that in patients receiving oral BPs was approximately 0.1%, which increased to 0.21% after more than 4 years of oral BP administration [11]. The incidence of BRONJ was found to be 10% in renal cell carcinoma patients with bone metastasis treated with the tyrosine kinase inhibitor sunitinib combined with BPs [12]. Furthermore, a cohort study reported that tooth extraction was associated with a 33-fold increased risk of BRONJ in cancer patients [13]. Thus, many dentists and oral surgeons do not remove teeth that should be extracted in patients exposed to BPs. However, these teeth can cause BRONJ via periodontitis or other dental diseases. Therefore, methods for patients receiving BPs are needed.

Although the etiology of BRONJ remains unclear, potential mechanisms have been proposed, including the over-suppression of bone turnover, suppression of angiogenesis, infection, soft tissue toxicity, and immune dysfunction [4,8,14]. It has been suggested that the bone-remodeling rate is high in the jaw [14]. Thus, BPs may have a greater impact on the health of the jawbones than on other bones. In addition, oral bacteria and thin gingiva may include the development of BRONJ only in the oral and maxillofacial region [4,8,14]. Although the number of patients with BRONJ is rapidly increasing, there is no definitive treatment or prevention for this disease.

Stage 1 BRONJ is defined as exposed and necrotic bone, or fistulae that probe into the bone, in patients who are asymptomatic. Stage 2 is defined as stage 1 with evidence of infection. Stage 3 is defined as stage 2 with more complications such as pathologic fracture, extra-oral fistulae, or others. Stages 1 and 2 are initially administered conservative treatments, including mouth rinse and antibiotics. Nonsurgical treatments only slow disease progression, but do not cure the disease [4]. A previous case study reported that initial stage cases progressed to the advanced stage in nearly half of the patients [15]. Stage 3 cases are commonly treated by surgery, such as sequestrectomy and resection [4,8]. However, several studies have suggested that the success rate of surgical treatment for BRONJ is only 60–86% [16–19]. Thus, many BRONJ patients do not receive active treatment. Therefore, new methods of treatment and prevention for BRONJ are needed.

Studies including small numbers of patients have been conducted to examine treatment with hyperbaric oxygen therapy [20], platelet-rich plasma [21], low-level laser irradiation [22], parathyroid hormone [23], and bone morphogenic protein [24]. Recent animal studies have demonstrated that intravenous injection (I.V.) of allogeneic multipotent mesenchymal stromal cells (MSCs) is effective for bone exposure in BRONJ-like animal models [25,26]. However, injected MSCs at the diseased area are relatively unstable [27]; thus, cells that do not engraft circulate throughout the body, resulting in pulmonary embolism and even death in some clinical cases and animal studies [28,29].

To overcome this problem, we developed cell sheet engineering using temperature-responsive culture dishes in which intact cells and extracellular proteins can be harvested as a sheet using simple temperature reduction [30,31]. Recent studies have confirmed that this technique is effective for the treatment of corneal dysfunction [32], myocardial infarction [33], esophageal ulcerations [34], diabetic ulcers [35], and periodontitis [36,37]. In this study, we investigated the effect of bone marrow-derived MSC sheet transplantation in a rat model of BRONJ-like disease.

2. Materials and methods

2.1. Animals and generation of a BRONJ rat model

Thirty-nine (39) SD rats (4-week-old females) were randomly divided into two groups, an untreated group (natural healing group in Fig. 6) (7 rats) and a BP-treated group (32 rats). Zoledronate (Zometa, 66 µg/kg; Novartis Pharma, Basel, Switzerland) and dexamethasone (5 mg/kg; Fuji Pharma, Tokyo, Japan) were subcutaneously administered to SD rats three times per week for 4 weeks in the BP-treated group. Two weeks after the first administration, the maxillary right first molars were extracted in each group. After an additional 2 weeks after extraction, the BRONJ model was confirmed in all cases of the BP-treated group (i.e., BP-treated rats) (Fig. 1A). All procedures were performed under general anesthesia with 4% isoflurane (Escaïne; Pfizer, New York, NY, USA) using a nasal mask connected to an inhalation anesthesia unit (Univentor 400 Anesthesia Unit; Univentor, Zejtun, Malta). All experimental protocols were approved by the animal welfare committee of Tokyo Women's Medical University.

2.2. Isolation and culture of rat bone marrow-derived MSCs

SD rats (4-week-old males) and BP-treated rats (4-week-old males) were used as the cell source. Bone marrow cells were flushed from the bone marrow cavity of femurs and tibiae with complete medium [α -Minimum Essential Medium (α -MEM; Life Technologies, Carlsbad, CA, USA) supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, and 10% fetal bovine serum (FBS; Japan BioSerum, Hiroshima, Japan)]. The cells were centrifuged for 5 min at 700g at room temperature and cultured in complete medium at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. One day after seeding, floating cells were removed and the medium was replaced with fresh medium. Adherent proliferating cells were subcultured using Trypsin-EDTA (0.25%; Life Technologies) (Fig. 1B).

2.3. Flow cytometry assay

One million cells were suspended in 100 µL of Dulbecco's phosphate-buffered saline (PBS) (Life Technologies) supplemented with 2% FBS containing 10 µg/mL of each specific antibody. To detect surface markers, fluorescein isothiocyanate (FITC)-coupled antibodies against CD11b, CD29 (BD Biosciences, Franklin Lakes, NJ, USA), CD31, CD45, and CD90 (AbD Serotec, Oxford, UK) were used. For the isotype control, fluorescein isothiocyanate-coupled non-specific mouse IgG1 (AbD Serotec), IgG2a, IgA, and non-specific hamster IgM (BD Biosciences) were substituted for the primary antibodies. After incubation for 30 min at 4 °C, the cells were washed with PBS supplemented with 2% FBS and suspended in 500 µL of PBS supplemented with 2% FBS for further analysis. Cell fluorescence was determined using a flow cytometer (Gallios; Beckman Coulter, Brea, CA, USA).

2.4. Colony-forming assay

MSCs at passage 3 were plated at a density of 1000 cells per 100-mm culture dish and cultured in complete medium. After 7 days, the cells were stained with 0.5% crystal violet (Kanto Chemical, Tokyo, Japan) in methanol for 5 min and washed twice with distilled water. Colonies larger than 3 mm in diameter and showing strong staining were then counted.

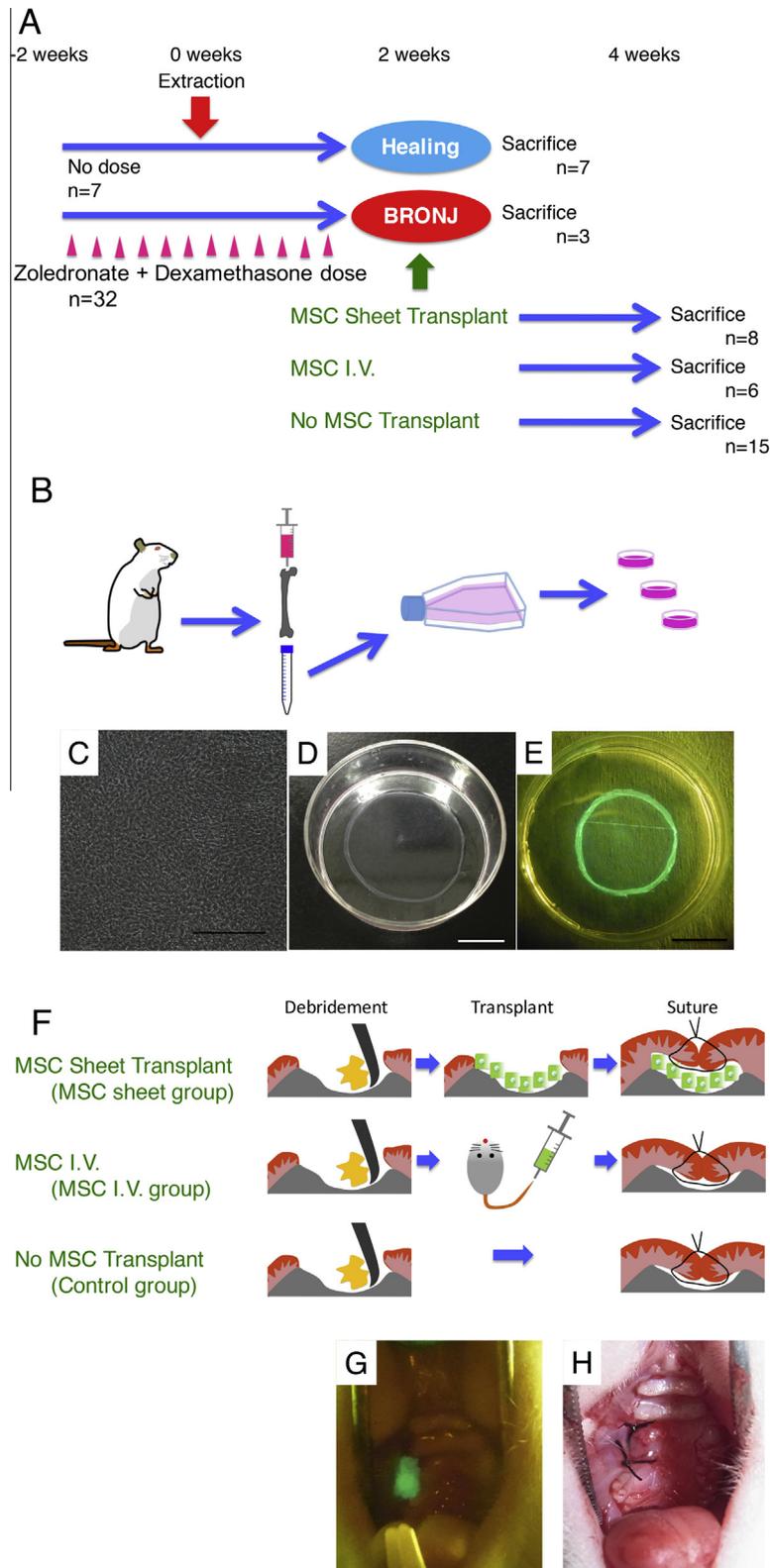


Fig. 1. (A) Flowchart of the timeline of the procedures performed in this study. Thirty-nine SD rats (4-week-old females) were randomly divided into two groups, an untreated group (7 rats) or a BP-treated group (32 rats). Zoledronate and dexamethasone were subcutaneously administered to SD rats three times per week for 4 weeks in the BP-treated group. Two weeks after the first administration, the maxillary right first molars were extracted from each group. Following additional 2 weeks after extraction, the BRONJ model was confirmed in all cases of the BP-treated group. Twenty-nine BP-treated rats were randomly divided into three groups, including an MSC sheet group (8 rats), MSC I.V. group (6 rats), or control group (15 rats). Two weeks after surgery, all rats in the three groups were sacrificed. (B) MSCs at passage 3 were seeded on temperature-responsive culture dishes (UpCell®) with 82 $\mu\text{g}/\text{mL}$ ascorbic acid and cultured for 7 days to produce MSC sheets. (C) Morphology of MSC sheet immediately before the transplantation. Scale bar: 500 μm (D, E) Macro images of EGFP-labeled MSC sheet. Scale bar: 10 mm (F) Schematic illustration of treatment methods in each group. After surgical debridement (only granulation tissue and food residues were removed without bone treatment), one MSC sheet (1.5×10^6 cells) was transplanted into a socket (MSC sheet group), single suspension of 1.5×10^6 MSCs were transplanted via rat tail vein (MSC I.V. group), or no cell transplantation (control group). The wounds in each group were closed using a silk suture. (G, H) Macro images of transplantation of EGFP-labeled MSC sheet and a suture in oral cavity of a rat model.

2.5. Differentiation assay

The differentiation assay was performed as previously described [38]. To study osteogenesis, 1000 cells were plated in a 100-mm culture dish and cultured for 7 days. The medium was replaced with osteoinductive medium, which consisted of complete medium supplemented with 82 µg/mL L-ascorbic acid phosphate magnesium salt (Wako, Osaka, Japan), 10 mM β-glycerophosphate (Sigma-Aldrich, St. Louis, MO, USA), and 10 nM dexamethasone (Dexart; Fuji Pharma, Tokyo, Japan), for an additional 14 days. The cells were fixed in 4% paraformaldehyde and stained with 1% alizarin red S solution (Wako), and the alizarin red S-positive colonies were counted. To assess adipogenesis, 1000 cells were plated in a 100-mm culture dish and cultured in complete medium for 7 days. The medium was replaced with adipogenic medium, which consisted of complete medium supplemented with 100 nM dexamethasone, 0.5 mM isobutyl-L-methylxanthine (Sigma-Aldrich), and 50 mM indomethacin (Wako), for an additional 14 days. The cells were fixed in 4% paraformaldehyde and stained with fresh oil red O solution (Wako), and the oil red O-positive colonies were counted.

2.6. Isolation of RNA and real-time PCR

Total RNA was isolated using a QIAshredder and the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). Next, cDNA was generated using the Superscript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR (StepOnePlus System; Applied Biosystems, Foster City, CA, USA) was performed using sequence-specific primers and probes (TaqMan Gene Expression Assays; Applied Biosystems) for receptor activator of nuclear factor κ-B ligand (RANKL) (Tnfsf11) (Rn00589289_m1), osteoprotegerin (OPG) (Tnfrsf11b) (Rn00563499_m1), and β-actin (ACTB) (4352340E). The mean fold changes in gene expression relative to β-actin were calculated using the delta CT method at each time point [39].

2.7. ELISA

The concentrations of vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) in the supernatants of cultured cells were detected using ELISA kits (R&D systems, Minneapolis, MN, USA) according to the manufacturer's recommendations.

2.8. Cell viability assay

The CellTiter 96 Aqueous One Solution cell proliferation assay (Promega, Madison, WI, USA) was used to evaluate cell viability. At passage 3, MSCs were seeded in 96-well plates at a concentration of 100 or 1000 cells/well in triplicate. The cells were cultured in complete medium for 2, 4, or 6 days. At the indicated time points, each well was incubated with 20 µL MTS assay solution for 1 h at 37 °C, and the absorbance was measured using a plate reader at an emission wavelength of 490 nm.

2.9. Preparation of MSC sheets

SD-Tg(CAG-EGFP) rats (4-week-old males) were used. MSCs at passage 3 were seeded onto temperature-responsive culture dishes (35 mm in diameter, UpCell®; Cell Seed, Tokyo, Japan) at a cell density of 2.5×10^5 cells/dish. The cells were cultured in complete medium supplemented with 82 µg/mL ascorbic acid (Wako) for 7 days. The temperature of the culture dish was reduced to room temperature, and the medium was aspirated to produce MSC sheets (Fig. 1B–E).

2.10. Transplantation of MSC sheets and intravenous injection of MSCs

Two weeks after extraction, bone exposure was confirmed at the extraction socket of BP-treated rats. Twenty-nine BP-treated rats were randomly divided into three groups; the MSC sheet group (one MSC sheet (1.5×10^6 cells) was transplanted into a socket, 8 rats), MSC I.V. group (single suspension of 1.5×10^6 MSCs was transplanted via rat tail vein, 6 rats), and control group (no cell transplantation, 15 rats). After surgical debridement (only granulation tissue and food residues were removed without bone treatment) in each group, transplantation was performed. The wounds were closed using a silk suture in all groups. The sutures were removed 1 week after surgery. Two weeks after surgery, all rats in the three groups were sacrificed (Fig. 1A, F–H).

2.11. Micro-CT analysis

Quantitative analysis of new bone in the extraction sockets was performed using an *in vivo* micro-computed tomography (CT) system (R_mCT2; Rigaku, Tokyo, Japan). The maxillary bones were scanned using micro-CT with an X-ray source of 90 kV/160 µA at 1, 14, and 28 days after extraction. Three-dimensional image-analysis software (TRI/3D-BON; Ratoc System Engineering, Tokyo, Japan) was used to construct three-dimensional images and perform bone morphometric analysis of the extraction sockets. For each socket, we measured the tissue volume, defined as the area surrounded by the inner wall of the extraction socket and the upper limit of the extraction socket as determined using lines passing through the bone crest of the alveolar septum. The calcified newly formed radiopaque finding in the extraction socket was differentiated from the highly calcified alveolar bone by manual tracing [40]. The calcified newly formed radiopaque finding/tissue volume ratio was calculated for 1, 14, and 28 days after extraction in each group.

2.12. Histological analysis

The maxillary tissues were removed, fixed in 70% ethanol, and embedded in glycol-methacrylate without decalcification. Next, sections (3 µm thick) were cut longitudinally using a microtome (Model 2255; Leica Microsystems, Wetzlar, Germany) and sections were stained with the hematoxylin and eosin or tartrate-resistant acid phosphatase (TRAP). The empty and occupied osteocyte lacunae within the cortex were counted, and the percentage was determined using the hematoxylin and eosin-stained sections. The osteoclast perimeter (#/mm²) was determined in the TRAP-stained sections.

2.13. Immunohistochemical analysis

Fresh-frozen maxillary sections (10 µm thick) were prepared using the Kawamoto film method [41]. After thawing, cryostat sections were incubated with 1% Block Ace blocking solution (DS Pharma Biomedical, Osaka, Japan) to reduce nonspecific background staining. The sections were then immunostained with a primary antibody for rat endothelial cell antigen-1 (RECA-1) (Abcam, Cambridge, MA, USA) and anti-CD146 (Abcam) for binding to endothelial cells at 4 °C overnight in humidified chambers. After primary antibody staining, the specimens were washed with PBS and incubated with Cy3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) or Alexa Fluor 647 Goat anti-Rabbit IgG [H+L] Antibody (Life Technologies) and mounted on coverslips with Prolong Gold.

2.14. Statistics

Pearson's chi-square test was used to evaluate the effect of each experiment. The mean differences between the two groups were analyzed by an unpaired two-tailed Student's *t*-test and the multi groups were analyzed by one-way analysis of variance, followed by Tukey's multiple range test by using JMP Pro 11.0.0 (SAS, Cary, NC, USA). A *P*-value of less than 0.05 ($P < 0.05$) was considered significant.

3. Results

3.1. Effect of BPs on MSCs

Flow cytometry analysis showed that both untreated and BP-treated MSCs were positive for the MSC-related markers, CD29 and CD90, and negative for the hematopoietic markers CD11b, CD31, and CD45 (Fig. 2A). Both untreated and BP-treated MSCs were positive in the colony-forming assay (31.6 ± 8.0 colonies and 8 ± 2.3 colonies, respectively) (Fig. 2B). Cells cultured in osteoinductive medium for 21 days showed alizarin red S-positive calcium deposits ($7.9 \pm 0.2\%$ and $8.0 \pm 0.2\%$, respectively) (Fig. 2C). Cells cultured in adipogenic medium for 21 days showed oil red O-positive lipid droplets ($8.8 \pm 0.5\%$ and $6.9 \pm 0.8\%$, respectively) (Fig. 2D). There were no significant differences between untreated and BP-treated MSCs in the differentiation assays. The proliferation of BP-treated MSCs was

significantly lower than that of untreated MSCs at all time points for both 100 cells and 1000 cells per well (Fig. 3A). The protein level of VEGF in the supernatant of BP-treated MSCs was significantly lower than that of untreated-MSCs and decreased in a time-dependent manner. There was no significant difference in HGF secretion between groups (Fig. 3B). The mRNA expression levels of RANKL and OPG were significantly decreased in BP-treated MSCs (Fig. 3C).

3.2. Rat model of BRONJ induced by zoledronate and dexamethasone

Two weeks after tooth extraction, incomplete wound healing and the presence of exposed bone were observed in all BP-treated rats (occurrence of bone exposure: 100%; 32/32). In contrast, the control group showed complete wound healing (occurrence of bone exposure: 0%; 0/7) (Fig. 4A, B). Micro-CT analysis revealed delayed and irregular bone healing in the extraction sockets of the BP-treated group, whereas the extraction sockets of the control group were nearly all radiopaque (Fig. 4C, D). Histological images showed a lack of epithelial lining in the extraction socket in the BP-treated group; however, complete epithelial coverage was observed in the control group (Fig. 4E). Histomorphometric analysis showed that the percentage of empty osteocyte lacunae significantly increased in the BP-treated group compared to the control group ($84.1 \pm 2.9\%$ compared to $13.6 \pm 4.1\%$) (Fig. 4F, G). TRAP staining showed that the average number of osteoclasts per mm^2 was significantly reduced in the BP-treated

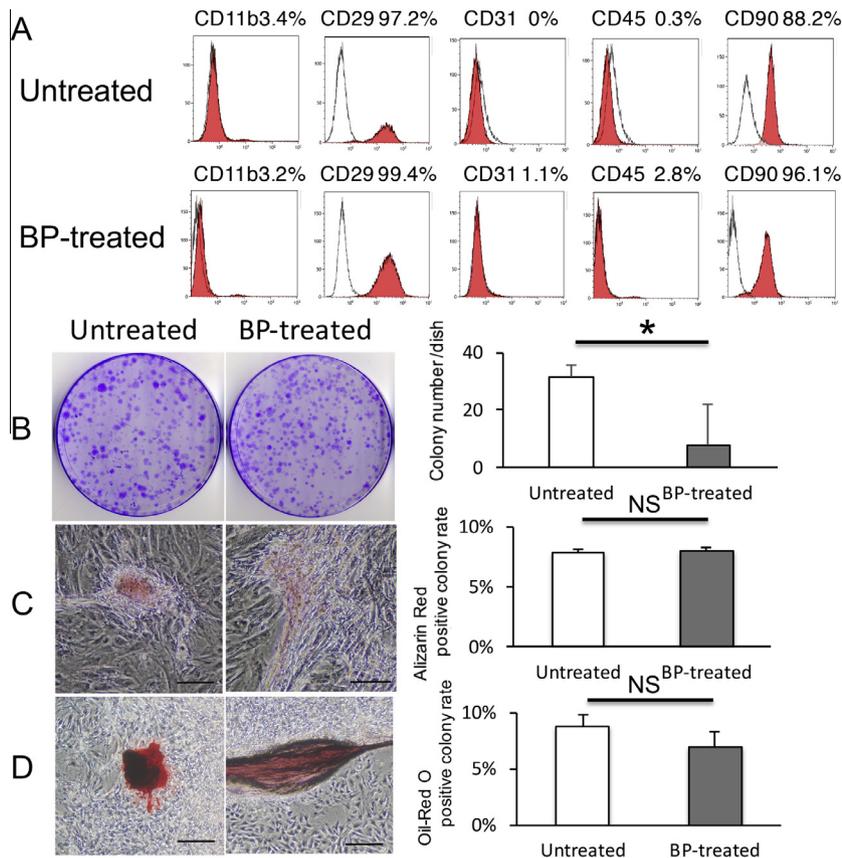


Fig. 2. Flow cytometry analysis, colony-forming assay, and differentiation assays. (A) Flow cytometry analysis showed that both untreated and BP-treated MSCs were positive for CD29 and CD90 and negative for CD11b, CD31, and CD45. (B) In the colony-forming assay, both untreated and BP-treated MSCs were positive (31.6 ± 8.0 colonies and 8 ± 2.3 colonies, respectively). (C) In the osteogenesis study, cells cultured in osteoinductive medium for 21 days showed alizarin red S-positive calcium deposits ($7.9 \pm 0.2\%$ and $8.0 \pm 0.2\%$, respectively). Scale bar: 100 μm . (D) In the adipogenesis study, cells cultured in adipogenic medium for 21 days showed oil red O-positive lipid droplets ($8.8 \pm 0.5\%$ and $6.9 \pm 0.8\%$, respectively). Scale bar: 50 μm . There were no significant differences between untreated and BP-treated MSCs in differentiation assays. MSCs were derived from 3 rats of each group in all experiments.

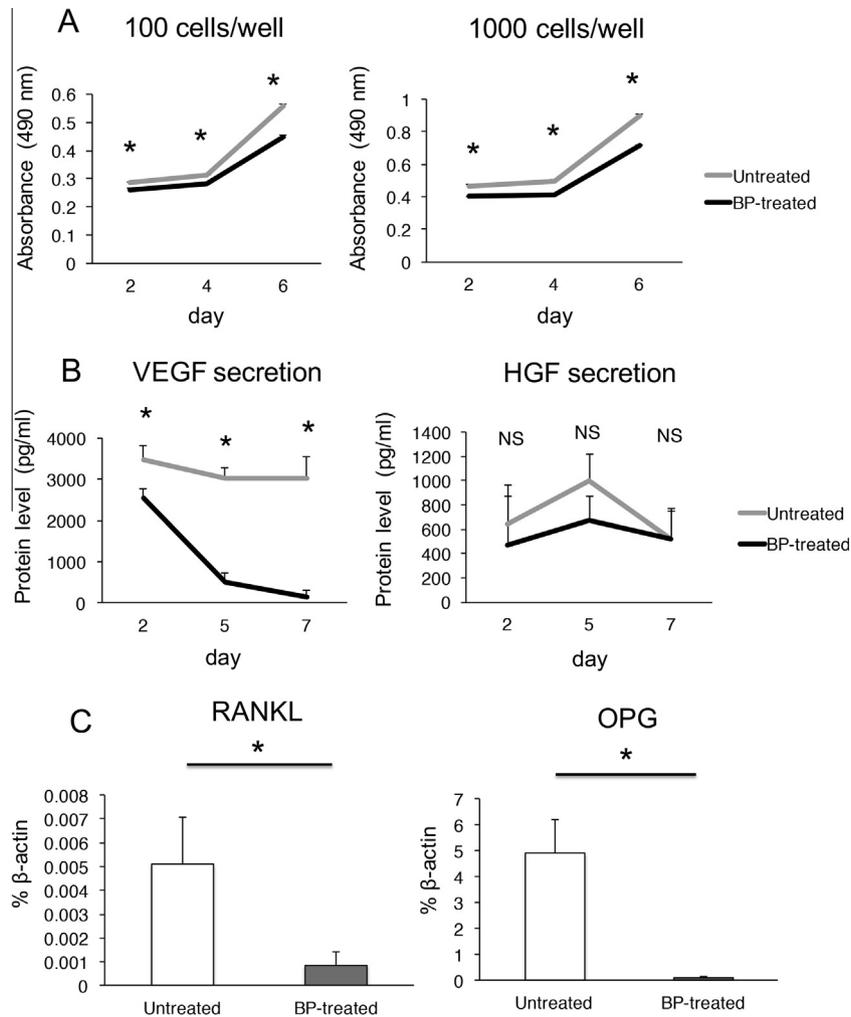


Fig. 3. Cell viability assay, ELISA, and quantitative real-time PCR. (A) The proliferation of BP-treated MSCs was significantly lower than that of untreated MSCs at all time points for both 100 cells and 1000 cells per well. (B) The protein levels of VEGF in the supernatant of BP-treated MSCs were significantly low and decreased in a time-dependent manner. There was no significant difference in HGF secretion between groups. (C) The mRNA expression levels of RANKL and OPG were significantly decreased in BP-treated MSCs. MSCs were derived from 3 rats from each group in all experiments.

group compared with the untreated control group (0 compared with 32 ± 7 cells/mm², respectively) (Fig. 4H, I).

3.3. Transplantation of the allogeneic enhanced green fluorescent protein (EGFP)-labeled MSC sheets

Two weeks after the transplantation, the MSC sheet group showed wound healing in most cases (occurrence of bone exposure: 12.5%; 1/8). In contrast, the control group and the MSC I.V. group showed exposed bone without soft tissue in most cases (occurrence of bone exposure: 80%; 12/15 and 100%; 6/6, respectively) (Fig. 5A, B). Micro-CT analysis showed bone regeneration in the extraction socket of the MSC sheet group. There was no significant difference in calcification volume in the extraction sockets between the three groups (Fig. 5C, D). Histological images showed that bone exposure healed with complete epithelial coverage in the MSC sheet group (Fig. 5E). Histomorphometric analysis also showed that the percentage of empty osteoclast lacunae was significantly reduced in the MSC sheet group compared to in the control group and MSC I.V. group ($31.0 \pm 6.3\%$ compared with $83.9 \pm 1.6\%$ and $89.1 \pm 1.9\%$, respectively) (Fig. 5F, G). TRAP staining showed that the average number of osteoclasts per mm² was significantly increased in the MSC sheet group compared to in the control group and MSC I.V. group (25.7 ± 3.4 compared with

6.3 ± 4.5 and 2.7 ± 1.3 cells/mm², respectively) (Fig. 5H, I). EGFP-positive cells (green) were observed in the transplanted submucosa of the MSC sheet group at 2 weeks after transplantation. Furthermore, immunohistochemical analysis showed that these cells were localized around RECA-1-positive blood vessels (red). A significantly number of newly formed blood vessels was observed in the MSC sheet group compared to in the control group, MSC I.V. group, and natural healing group (106 ± 9.6 compared with 40 ± 5.3 , 62 ± 10.2 , and 61 ± 6.1 vessels/mm², respectively) (Fig. 6A and B). Most CD146-positive cells (red) were observed inside the EGFP-positive cells (green) and along the surface of the vessels. Some CD146-positive cells were merged with EGFP-positive cells (Fig. 6C).

4. Discussion

The use of oral BPs in patients with osteoporosis is effective for reducing the fracture risk [2]. In addition, intravenous BPs in patients with multiple myeloma, breast cancer, and other solid tumors remarkably improve the quality of life by reducing skeletal-related events with bone metastasis [3]. However, several reports have indicated that BPs are strongly related to osteonecrosis development in the jaws. The risk of BRONJ among cancer

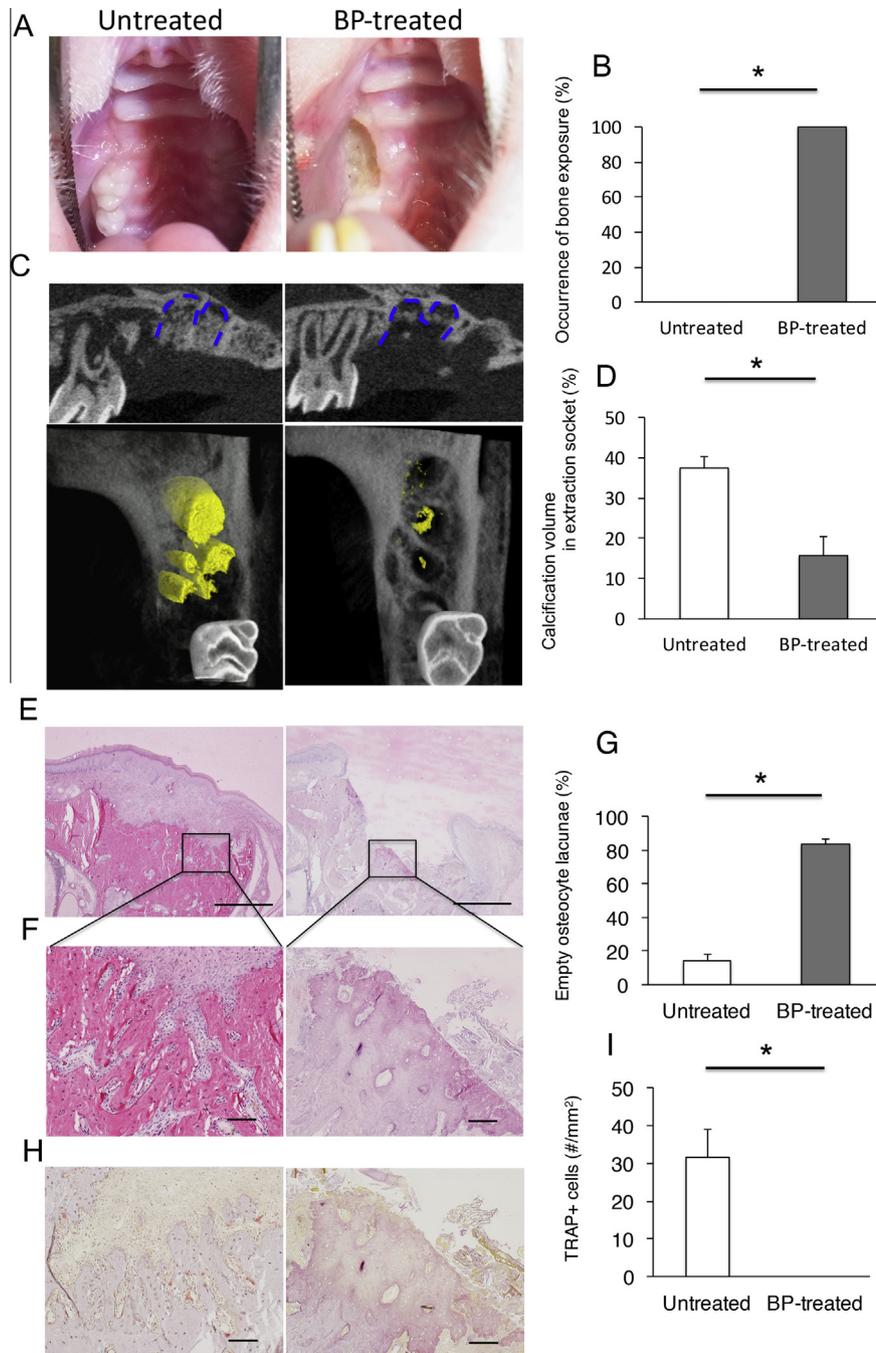


Fig. 4. Rat model of BRONJ-treated zoledronate and dexamethasone. (A, B) Two weeks after tooth extraction, incomplete wound healing and exposed bone were observed in all BP-treated groups (occurrence of bone exposure: 100%; 32/32). (C) Micro-CT analysis showed delayed and irregular bone healing in the extraction sockets of the BP-treated group. (D) Calcification volume was significantly reduced in the extraction socket of the BP-treated group. (E) Histological images revealed a lack of epithelial lining in the extraction socket. Scale bar: 1 mm. (F) Enlarged images of the square areas in E. Scale bar: 100 μ m. (G) The percentage of empty osteocyte lacunae was significantly increased in the BP-treated group. (H, I) TRAP staining showed that the average number of osteoclasts per mm² was significantly reduced in the BP-treated group. Scale bar: 100 μ m.

patients exposed to zoledronate is approximately 1% according to systematic reviews and randomized controlled trials [9,10]. The prevalence of BRONJ in patients receiving long-term oral BP therapy was reported to be approximately 0.1% [11]. Recently, BRONJ-like models have been established in various animals [25,26,42–44]. The administration of BPs combined with dexamethasone induced BRONJ-like models with exposed bone in some studies [25,43,44], is clinically relevant because most cancer patients receive multiple immunosuppressive drugs, including dexamethasone and chemotherapeutic agents [45]. In this study,

BRONJ-like models were successfully produced in all cases via the administration of zoledronate combined with dexamethasone and tooth extraction.

Recent studies suggested that allogeneic MSCs I.V. was effective for treating BRONJ-like disease models [25,26]. MSCs I.V. suppressed the number of Th17 cells and restored the number of regulatory T cells in the peripheral blood of BRONJ-like mice [25]. In addition, other studies conducted autologous bone marrow stem cells transplantation with platelet-rich plasma to treat BRONJ in patients [46,47]. However, animal studies have indicated that

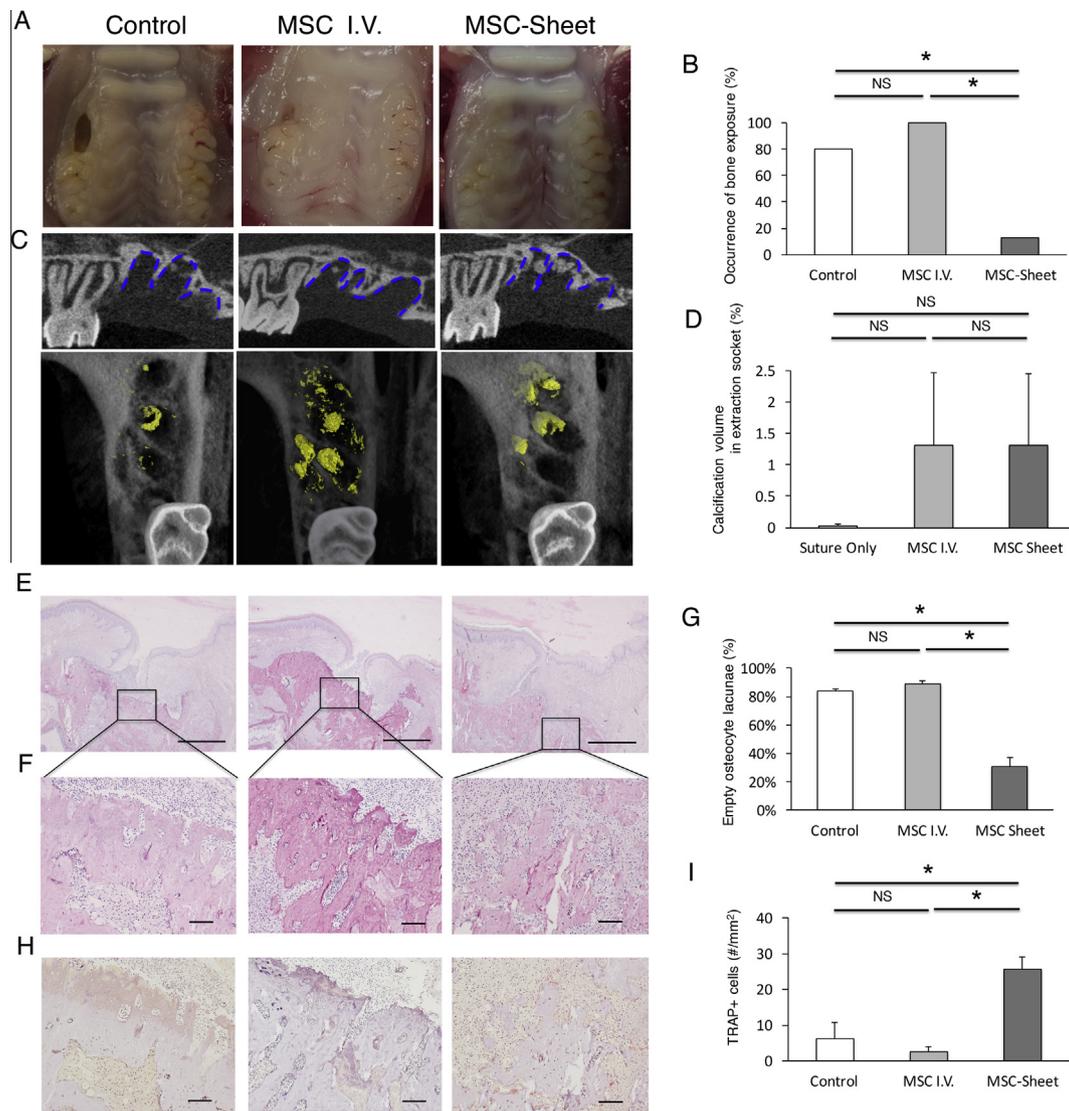


Fig. 5. Transplantation of allogeneic MSC sheet and intravenous injection of allogeneic MSC in the rat model. (A, B) Two weeks after transplantation, the MSC sheet group showed wound healing in most cases (occurrence of bone exposure: 12.5%; 1/8). In contrast, the control group and MSC I.V. group showed bone exposure without soft tissue in most cases (occurrence of bone exposure: 80%; 12/15 and 100%; 6/6, respectively). (C) Micro-CT analysis showed bone regeneration in the extraction socket of the MSC sheet group. (D) There was no significant difference in calcification volume in the extraction sockets of the three groups. (E) Histological images showed that bone exposure healed with epithelial coverage. Scale bar: 1 mm. (F) Enlarged images of the square areas in E. Scale bar: 100 μ m. (G) The percentage of empty osteocyte lacunae was significantly reduced in the MSC sheet group. (H, I) TRAP staining showed that the average number of osteoclasts per mm² was significantly increased in the MSC sheet group. Scale bar: 100 μ m.

MSCs frequently congregate within the pulmonary circulation after I.V [29]. In addition, clinical MSCs I.V. has caused the death of some patients due to pulmonary thromboembolism [28]. Many patients with intractable BRONJ are treated for cancer. This suggests that MSCs I.V. accelerate human breast tumor growth by generating cytokine networks that regulate the cancer stem cell population [48]. MSCs I.V. may also provide a tumor microenvironment that promotes the proliferation of gastric cancer cells [49]. Thus, it is possible that MSCs I.V. promote cancer progression in BRONJ patients. Moreover, because MSCs may increase osteoclasts in the entire body, they may also cause cancer metastasis, which is suppressed by BPs. Thus, local administration of MSCs may be safer than a systemic injection. In our laboratory, cell sheet engineering was developed using temperature-responsive culture dishes grafted with poly-*N*-isopropylacrylamide [30,31]. Cell sheets can be harvested without the use of enzymes by reducing the temperature to below 32 $^{\circ}$ C, and cell-membrane proteins and extracellular proteins are retained. Thus, we attempted to treat BRONJ using

topical cell sheet transplantation, which was shown to be effective for various diseases.

We first investigated the effect of BPs with dexamethasone on MSCs. MSCs were harvested from both BP-treated and untreated rats. In our study, both the multipotency and surface markers [50] of MSCs showed no significant differences from the controls. In contrast, both the colony-forming activity and the proliferation of BP-treated MSCs were significantly lower than that of untreated MSCs at every time point and the secretion of VEGF was downregulated in BP-treated MSCs. Moreover, the gene expression of both RANKL and OPG were significantly decreased. A recent report showed that BPs decreased the proliferation of human umbilical vein endothelial cells in a time-dependent manner [51]. In addition, BPs were reported to significantly inhibit VEGF expression in estrogen receptor-positive human breast cancer cells and the protein levels of VEGF were decreased in cancer patients treated with BPs [52,53]. *In vitro* studies showed that BPs enhance the OPG gene expression [54]. In contrast, it was reported that

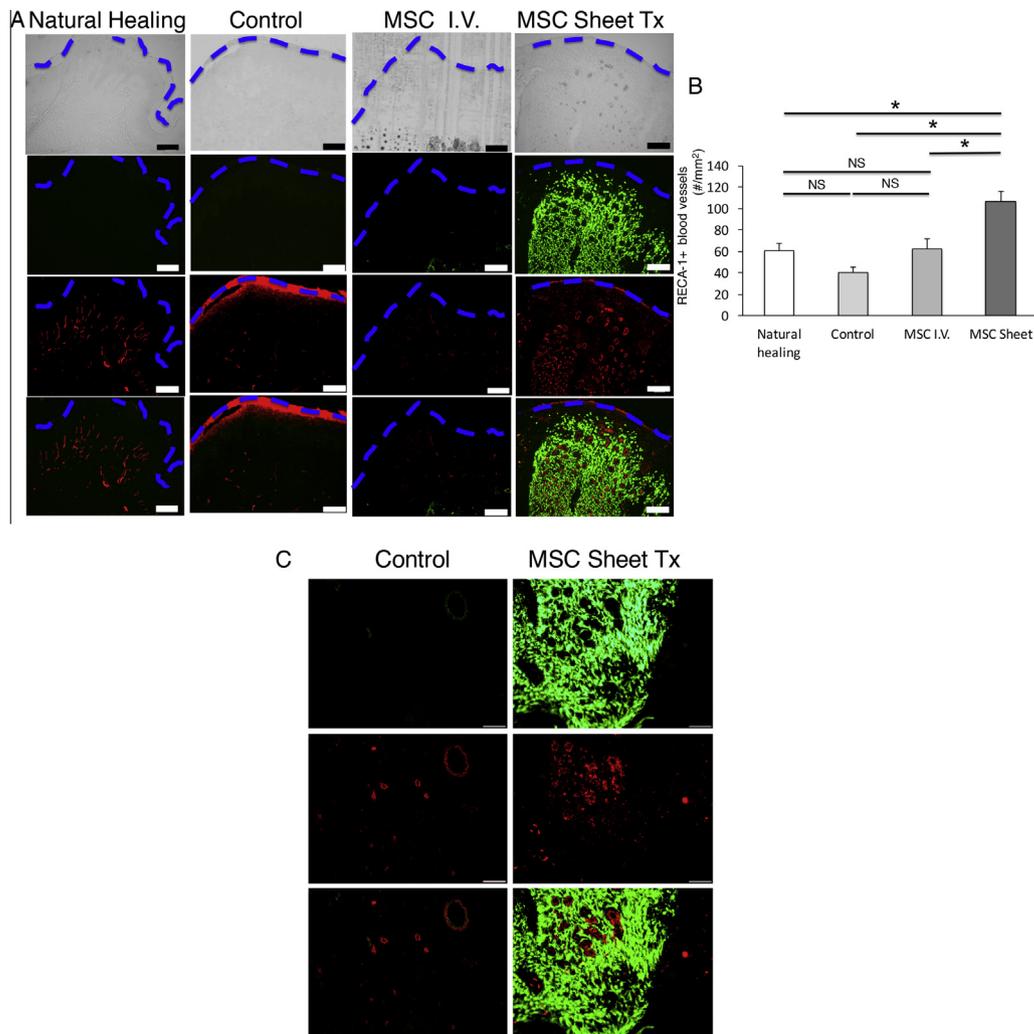


Fig. 6. Immunohistochemical analysis. (A) EGFP-positive cells (green) were observed in the transplanted sub-mucosa of the MSC sheet group at 2 weeks after transplantation. Furthermore, immunohistochemical analysis showed that these cells were localized around rat endothelial cell antigen-1-positive blood vessels (red). Scale bar: 200 μ m. (B) The number of newly formed blood vessels was significantly higher in the MSC sheet transplant group than in the control group, MSC I.V. group, and natural healing group. (C) Most CD146-positive cells (red) were detected inside the EGFP-positive cells (green) and along the surface of vessels. Some CD146-positive cells were merged with EGFP-positive cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

dexamethasone significantly decreases the expression of both OPG and RANKL in human osteoblastic cells [55]. In this study, we administered both BPs and dexamethasone to induce the BRONJ-like phenotype. Further investigation is needed to determine the effect of combined use of BPs and dexamethasone on the gene expression in MSCs taking into the medical history of MSC donors. Based on the reductions in proliferation, VEGF secretion, and RANKL expression, the use of MSCs derived from BRONJ patients is not superior to the use of cells from healthy donors. Therefore, in this study, MSCs were derived from allogeneic EGFP rats.

In this study, allogeneic MSC sheet transplantation significantly promoted the mucosal healing of BRONJ-like model rats compared to both the control group and MSC I.V. group. Two weeks after transplantation, the transplanted MSC sheet remained at the transplanted site. In contrast, few I.V. MSCs were observed at the transplanted site. Compared with naturally healing rats, newly formed blood vessels were surrounded by transplanted MSCs. Furthermore, most CD146-positive cells were observed inside transplanted MSCs and along the surface of the vessels. Some CD146-positive cells were merged with transplanted MSCs. It was reported that MSCs may differentiate into pericytes and

secrete many various bioactive molecules via their local trophic and immunomodulatory activities [56]. In our study, both differentiated MSCs (into pericytes) and undifferentiated MSCs were present in the wound healing area at 2 weeks after the transplantation. Additionally, MSCs may stimulate angiogenesis by secreting VEGF and HGF and stabilize new blood vessels by differentiating into the pericyte phenotype [57]. Our study showed that VEGF and HGF were secreted into the supernatant of MSCs, suggesting that angiogenesis and wound healing were promoted in BRONJ-like model rats. Similar to the transplanted MSCs, osteoclast precursor cells can differentiate into osteoclasts in response to RANKL secretion from MSCs, accelerating bone turnover. In addition, the immunomodulatory and anti-inflammatory activities of MSCs have been reported in numerous animal models and clinical trials [25,26,56,58] (Fig. 7).

Although it has been reported that MSCs I.V. is effective for healing exposed bone in mouse and pig models of BRONJ [25,26], our results did not show a significant difference compared with the control group in the MSC I.V. group. The stability of injected MSCs may be low, as observed in our results. These discrepancies may arise from the difference of experimental conditions and species used. The EGFP signal was strongly observed in the MSC sheet

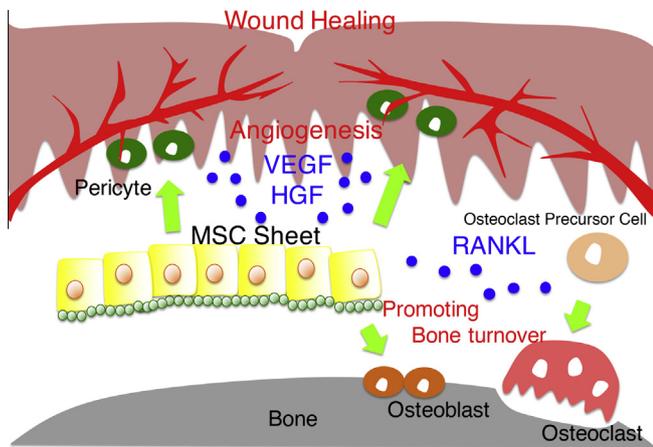


Fig. 7. Schematic hypothesis of function of MSC sheet transplantation. Our experiments suggest that both VEGF and HGF are secreted from MSCs at the wound, and subsequent angiogenesis and wound healing are promoted in BRONJ-like model rats. Furthermore, osteoclast precursor cells can differentiate into osteoclasts via RANKL secreted from the MSCs, together with the transplanted MSCs, accelerating bone turnover.

transplantation group throughout the experiments. In addition, taking the increased proliferation and metastasis of cancer cells into account, intravenous infusion of MSCs is expected to be impossible for a clinical application. In contrast, the local administration of the MSC sheet may only minimally affect general symptoms and MSCs remain at the diseased area to retain cell functions. Therefore, the MSC sheet is a promising alternative approach for treating BRONJ. In future studies, we will establish a larger animal model of BRONJ to further analyze the clinical application of the MSC sheet.

5. Conclusion

MSC sheet transplantation showed significant wound healing effects compared to MSC intravenous injection or sham surgery in a rat model of BRONJ.

Conflicts of interest

Teruo Okano is a founder and director of the board of CellSeed, Inc. and holds technology licensing and patents from Tokyo Women's Medical University. Teruo Okano is a stakeholder of CellSeed, Inc. Tokyo Women's Medical University receives research funds from CellSeed, Inc. The other authors disclosed no financial relationships relevant to this publication.

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