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

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A B S T R A C T

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/mm^2, 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 (Escaine; 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 tibias 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% CO_2. 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.

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