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Evaluation of the therapeutic effects of conditioned media from mesenchymal stem cells in a rat bisphosphonate-related osteonecrosis of the jaw-like model

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ABSTRACT

Bisphosphonate-related osteonecrosis of the jaw (BRONJ) is defined as an exposed necrotic bone in the oral cavity that does not heal after appropriate intervention for >8 weeks with present or previous bisphosphonate treatment in the absence of radiotherapy. Until now, although several risk factors, including invasive dental procedures, infection, mechanical trauma to the jawbone, and concomitant use of immunosuppressive and chemotherapy drugs have been implicated in the etiology of BRONJ, its underlying mechanisms and treatments remain largely unknown. A study recently showed that intravenous administration of mesenchymal stem cells (MSCs) improved BRONJ, and it was hypothesized that paracrine effects by secretomes from MSCs are the main constituent. Here we used rat BRONJ models to examine the therapeutic effects with serum-free conditioned media from human MSCs (MSC-CM), including various secretomes. We showed that MSC-CM has protected rat MSCs and rat osteoclasts. MSC-CM enhanced the expression of osteogenic-related genes and neovascularization-related genes by real-time reverse-transcriptase polymerase chain reaction analysis in in vitro study. In in vivo study, 5-week-old Wistar/ST male rats received zoledronate (35 μg/kg/week) and dexamethasone (1 mg/kg/day) subcutaneously for 2 weeks. Unilateral maxillary molars were then extracted. Two weeks later, rats were divided into non-treatment, serum-free Dulbecco's modified Eagle's medium, and MSC-CM groups. In the MSC-CM group, the open alveolar sockets in 63% of the rats with BRONJ healed with complete soft tissue coverage and socket bones, whereas the exposed necrotic bone with inflamed soft tissue remained in the other groups. Histological analysis showed new bone formation and the appearance of osteoclasts in the MSC-CM group. Osteoclasts were significantly reduced in the non-treatment group. Thus, we concluded that the antiapoptotic and antiinflammatory effects of MSC-CM dramatically regulated the turnover of local bone and indicated therapeutic effects on BRONJ.

Introduction

Bisphosphonates (BPs) were originally developed as a drug for the treatment of bone-resorbing diseases, such as multiple myeloma and bone metastasis, whose typical origins are breast cancer and prostate cancer, as well as tumor-related hypercalcemia [1–4], and then to prevent pathological fracture in patients with osteoporosis. Lately, reports in the literature have suggested that a rare but potentially severe side-effect is BP-related osteonecrosis of the jaw (BRONJ) [5], defined as an exposed bone in the maxillofacial region that persists for more than 8 weeks in patients with present or previous BP treatment without a history of radiation therapy to the jaw [6]. Specific target cells of BPs are osteoclasts and mesenchymal stem cells (MSCs) (or osteoblasts), which play a central role in physiological and pathological bone formation and bone resorption [7–10].

Abbreviations: BPs, bisphosphonates; BRONJ, bisphosphonate-related osteonecrosis of the jaw; MSCs, mesenchymal stem cells; MSC-CM, serum-free conditioned media from human MSCs; Zol, zoledronate; Dex, dexamethasone; rOSCs, rat osteoclasts; *Runx2*, *Runt-related transcription factor 2*; *OCN*, *osteocalcin*; *vegf-a*, *vascular endothelial growth factor-a*; *RANKL*, *receptor activator of nuclear factor kappa-B ligand*; *OPG*, *osteoprotegerin*; IGF-1, insulin-like growth factor-1; MCP-1, monocyte chemoattractant protein-1; VEGF-A, vascular endothelial growth factor-A; Ang, Angiogenin; IL-6, interleukin-6; M-CSF, macrophage colony-stimulating factor.

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Risk factors for BRONJ include invasive dental procedures, infections, mechanical trauma to the jawbone, and length of exposure to BPs, as well as the concomitant use of immunosuppressive and chemotherapeutic drugs [6,11]. Because most patients with cancer were receiving multiple immunosuppressant drugs, including dexamethasone (Dex) and chemotherapeutic agents, and thus experiencing some degree of impaired immunity, it is likely that immunosuppression contributes to an increased susceptibility to BRONJ.

Patients with BRONJ present various jaw symptoms, including pain, swelling, infection and, in some severe cases, pathologic fracture [5,6,11]. Histologically, several tissue alterations are observed in BRONJ, including necrotic bone honeycombed with residual vital bone, inflammatory cellular elements, and hypernucleated osteoclasts and fibrous tissues [10–12]. Many attempts to control this disorder have been unsuccessful, and standard osseous sequestrectomy usually results in further enlargement of the bone defects [13,14]. Therefore, conservative non-surgical approaches to the management of BRONJ have been recommended that slow its deterioration but do not cure the disease [15–17]. The development of an effective approach to the prevention and treatment of BRONJ is an urgent issue for patients using BPs.

Some studies have reported that an intravenous injection of MSCs improved BRONJ [18,19], but tumorigenesis of the cells is also possible [20,21]. Moreover, recent studies of MSC transplantation demonstrated that the implanted MSCs did not survive for a long time [22]. Furthermore, it has been established that MSCs secrete a variety of growth factors and cytokines [23–28], and the paracrine effects of growth factors and cytokines secreted from the implanted MSCs may promote tissue repair or have antiapoptotic effects [23–28] and prevent BRONJ [18,19]. The paracrine factors secreted by MSCs can accumulate in conditioned media during cell culture [23–28]. The serum-free conditioned media from human MSCs (MSC-CM) have been reported to serve multiple positive functions [29,30]. In particular, study results to date have supported the theory that MSC-CM is included in the paracrine factors important for the turnover of local bone status [23–28].

In late years various rat BRONJ-like models are reported [31–34]. Here we made a rat BRONJ-like model, using zoledronate (Zol) and Dex which are used clinically widely, that recapitulates major clinical and radiographic manifestations of the human disease, including its characteristic features of delayed healing displayed orally as an open alveolar socket without mucosal coverage, exposed necrotic bone or sequestra, increased inflammatory infiltrates, osseous sclerosis, and radiopaque alveolar bone in the jaw.

In this in vitro study, to understand how MSC-CM alters the turnover of local bone status and the inflammatory response of alveolar socket healing, we investigated the effects on MSCs and osteoclasts of MSC-CM. In an in vivo study, we investigated whether MSC-CM injection can have therapeutic effects on BRONJ by using a rat BRONJ-like model.

Materials and methods

Cell preparation

All animal experiments undertaken in this study were performed in strict accordance with the protocols approved by the Guidelines for Animal Experimentation of the Nagoya University School of Medicine (approval nos. 25374 and 26063). Human MSCs (hMSCs) were purchased from Lonza, Inc. (Walkersville, MD, USA) and cultured in MSC basal medium (Lonza, Inc.) containing MSCGM SingleQuots (Lonza, Inc.) at 37 °C in 5% CO_2/95% air. After primary culture, the cells were subcultured at a density of approximately 1×10^4 cells/cm^2. hMSCs at the third to the sixth passages were used for experiments.

Rat MSCs (rMSCs) were isolated from 7-week-old Wistar/ST male rats (Japan SLC, Shizuoka, Japan) as previously reported [35]. Briefly, donor rats were sacrificed, and the femora were dissected out. Under sterile conditions, the edge of each bone was cut, Dulbecco's modified Eagle's medium (DMEM; Gibco, Rockville, MD, USA) was injected into the bone marrow using an 18-gauge syringe and the bone marrow cells were flushed out to the opposite side; this procedure was repeated several times. The marrow was then seeded into each tissue culture flask in DMEM containing an antibiotic–antimycotic solution (100 units/ml penicillin G, 100 mg/ml streptomycin, and 0.25 mg/ml amphotericin B; Gibco), and the medium was supplemented with 10% fetal bovine serum (FBS). Three days after seeding, floating cells were removed and the medium was replaced with fresh medium. The adherent, spindle-shaped cells were passaged when the cells approached confluence. Adherent cells were collected with trypsin/EDTA, resuspended in fresh medium and transferred to new flasks at a density of 1×10^4 cells/cm^2.

After Zol (35 μg/kg/week) (ZOMETA®, Novartis Pharmaceuticals, Tokyo, Japan) and Dex (1 mg/kg/day) (DECADRON®, Merck & Co., Inc., Tokyo, Japan) were subcutaneously injected into 5-week-old Wistar/ST male rats for 2 weeks [30,35], rMSCs were isolated by the same abovementioned method (Zol rMSCs). The pluripotency of cells obtained for differentiation into classic mesenchymal lineage cells, including osteoblasts, adipocytes, and chondrocytes, was verified by previously reported methods. These cells were used in this study as rMSCs or Zol rMSCs [37].

Preparation of conditioned media

hMSCs that were 80% confluent were re-fed with serum-free DMEM [DMEM(−)] containing antibiotic–antimycotic solution. The cell-cultured conditioned media were collected after 48 h of incubation. The collected cultured conditioned media were defined as MSC-CM and were stored at −80 °C before being used for the following experiments.

Enzyme-linked immunosorbent assay (ELISA) analyses

The levels of insulin-like growth factor (IGF)-1, monocyte chemoattractant protein (MCP)-1, vascular endothelial growth factor-A (VEGF-A), angiogenin (Ang), interleukin (IL)-6, macrophage colony-stimulating factor (M-CSF), bone morphogenetic protein (BMP)-2, and parathyroid hormone-related peptide (PTHrP) in MSC-CM were investigated by ELISA. The concentrations of these factors were measured with a Human Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA) and with a PTH-RP (1–34) (human, rat, mouse) ELISA (DRG International, Inc., Springfield, USA) according to the manufacturer's instructions.

Cell viability assay

BPs are strong inhibitors of MSC or mature osteoclast functions, such as bone resorption and survival [7–10]. To test whether Zol-induced inhibition in rMSCs and rat osteoclasts (rOSCs) was improved by MSC-CM, we performed a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

rMSCs and rOSCs (COSMO BIO COMPANY, LIMITED, Tokyo, Japan) were cultured at a density of 1×10^4 and 3×10^4 cells/well, respectively, in 96-well culture plates at 37 °C in 5% CO_2/95% air. When they became 80% confluent, they were treated with various concentrations of Zol or MSC-CM for 6 h, 24 h, 48 h or 72 h. After the indicated culture time, MTT assay was performed by incubation of cells with 0.2% MTT solution (Cell Counting Kit, DOJINDO, Tokyo, Japan) at 37 °C for 4 h. The resulting color was then analyzed by measuring the absorbance at 450 nm (A450), and A450 corresponded to the viability of the cells [9]. Then, to investigate the influence of the MSC-CM concentration changes, we performed similar experiments using rMSCs and rOSCs. We prepared the solutions which diluted the concentration of MSC-CM undiluted solution (×1), double dilution (×1/2), and 4 fold dilution (×1/4). When rMSCs and rOSCs became 80% confluent, they were treated with