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Prevalence of bisphosphonate-related osteonecrosis of the jaw-like lesions is increased in a chemotherapeutic dose-dependent manner in mice

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ABSTRACT

Bisphosphonate-related osteonecrosis of the jaw (BRONJ) worsens oral health-related quality of life. Most BRONJ occurs in multiple myeloma or metastatic breast cancer patients treated with bisphosphonate/chemotherapeutic combination therapies. Cyclophosphamide (CY), an alkylating chemotherapeutic drug, is used to treat multiple myeloma, although its use has been recently reduced. The aim of this study was to clarify the effects of CY dose on tooth extraction socket healing when CY is used with or without bisphosphonate in mice. Low-dose CY (50 mg/kg; CY-L), moderate-dose CY (100 mg/kg; CY-M), high-dose CY (150 mg/kg; CY-H), and bisphosphonate [Zometa (ZA): 0.05 mg/kg] were administered for 7 weeks. Each dose of CY and ZA in combination was also administered for 7 weeks. Both maxillary first molars were extracted at 3 weeks after the initiation of drug administration. Euthanasia was performed at 4 weeks post-extraction. Gross wound healing, microcomputed tomography analysis, histomorphometry, and immunohistochemistry were used to quantitatively evaluate osseous and soft tissue wound healing of tooth extraction sockets. ZA monotherapy induced no BRONJ-like lesions in mice. CY monotherapy rarely induced open wounds, though delayed osseous wound healing occurred in a CY dose-dependent manner. In contrast, CY/ZA combination therapy prevalently induced BRONJ-like lesions with compromised osseous and soft tissue healing in a CY dose-dependent manner. Interestingly, anti-angiogenesis was noted regardless of CY dose and ZA administration, even though only CY-M/ZA and CY-H/ZA combination therapies induced BRONJ-like lesions. Our findings suggest that high-dose CY may be associated with the development of BRONJ following tooth extraction only when CY is used together with ZA. In addition to anti-angiogenesis, other factors may contribute to the pathoetiology of BRONJ.

1. Introduction

Nitrogen-containing bisphosphonates bind and inhibit a key enzyme of the intracellular mevalonate pathway, thereby resulting in apoptosis of osteoclasts. In general, oral bisphosphonates are used to treat osteoporosis, whereas intravenous bisphosphonates are utilized to reduce the risks of skeletal-related events such as pathological vertebral fracture and bone pain in oncology or metastasis patients [1]. However, in 2003, bisphosphonate-related osteonecrosis of the jaw (BRONJ) was first reported to occur following tooth extraction in patients taking intravenous bisphosphonates [2]. BRONJ is defined as “exposed bone or bone that can be probed through an intraoral or extraoral fistula in the maxillofacial region that has persisted for more than 8 weeks” [3]. The prevalence of BRONJ is rare, occurring in 0.05% of oral bisphosphonate users following tooth extraction, and 1.6%–14.8% of intravenous bisphosphonate users following tooth extraction [4]. A systematic review reported that BRONJ most frequently occurs in multiple myeloma or metastatic breast cancer patients taking intravenous bisphosphonates [5]. Tooth extraction (61.7%), dental implant treatment (3.9%), dental surgery (7.2%), periodontal disease (5.0%), prosthetic trauma such as ill-fitting removable prostheses (7.4%), and spontaneous cases (14.8%) are triggering factors of BRONJ [5], although all dental therapies including tooth cleaning, root canal filling, and prosthodontic treatment are not contraindicated in patients taking bisphosphonates. BRONJ worsens oral health-related quality of life [6], which results in negative effects on daily life and social activities. However, the exact mechanism of BRONJ has not been clear. Moreover, definitive treatment strategies for BRONJ have not been developed due to its uncertain mechanisms. Therefore, elucidation of the pathoetiology and pathophysiology of BRONJ is required.

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Multiple myeloma, which represents 1% of all cancers and approximately 10% of all hematologic malignancies, occurs in 20,000 people in the United States every year [7]. Moreover, the age-adjusted 5-year relative survival rate increased from 39.8% to 53.2% [7]. The increase in survival rate of multiple myeloma patients is due to the development of therapeutic strategies. A current review reported that thalidomide, lenalidomide, bortezomib, carfilzomib, pomalidomide, cyclophosphamide, melphalan, dexamethasone, and prednisone are used to treat multiple myeloma solely or adjunctively [8]. Generally, cyclophosphamide or melphalan are utilized for the treatment of multiple myeloma in combination with intravenous bisphosphonates that reduce the risks of skeletal complications [9]. The use of cyclophosphamide or melphalan in combination with prednisone, thalidomide, bortezomib, or carfilzomib was introduced as the major treatment regimens for multiple myeloma in 2016 [8], whereas at the 2017 American Society of Hematology Annual Meeting, the use of cyclophosphamide and melphalan were reportedly decreased [10]. Different doses of cyclophosphamide are administered to multiple myeloma patients depending on the patient's age and tolerability [9]. However, the effects of different doses of cyclophosphamide on tooth extraction socket healing, with or without bisphosphonate therapy, are not fully understood.

The aims of the present study were 1) to clarify the effects of cyclophosphamide dose on tooth extraction socket healing, and 2) to investigate the effects of each dose of cyclophosphamide and bisphosphonate combination therapy on osseous and soft tissue wound healing of tooth extraction sockets in C57BL6/J mice.

2. Materials and methods

2.1. Animals, tooth extraction, and drug therapies

Eight- to twelve-week-old male C57BL/6J mice were used (CLEA Japan Inc., Osaka, Japan). The duration of drug therapies was 7 weeks. Both maxillary first molars were extracted at 3 weeks after the start of drug therapy. All mice were euthanized at 4 weeks post-extraction. Subcutaneous injection of zoledronate (Zometa; Novartis, Stein, Switzerland) was performed at 0.05 mg/kg twice a week for 7 weeks (ZA, n = 7). Intraperitoneal injection of cyclophosphamide (C7397; Sigma-Aldrich, St. Louis, MO, USA) was carried out at 50 mg/kg (low dose; CY-L, n = 7) and 100 mg/kg (moderate dose; CY-M, n = 7) twice a week for 7 weeks. Additionally, 150 mg/kg of CY (high dose; CY-H, n = 7) was intraperitoneally injected twice and once a week before and after tooth extraction for 7 weeks, respectively. ZA was also subcutaneously injected for 7 weeks in combination with CY-L, CY-M, and CY-H (CY-L/ZA, CY-M/ZA, and CY-H/ZA, respectively, n = 7/each group). Saline was used as a control (VC, n = 7) (Fig. 1). Animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Nagasaki University, with approval from the Ethics Committee for Animal Research.

2.2. Microcomputed tomography (MicroCT)

After euthanizing mice, right maxillae including tooth extraction sites were dissected and fixed in 10% neutral buffered formalin for 24 h. They were scanned using microCT at 20-μm voxel resolution and 90-kV tube voltage (R_mCT2; Rigaku Co. Ltd., Tokyo, Japan) [11]. Segmentation and reconstruction of tooth extraction sockets were performed with a semi-manual contouring method using TRI/3D-Bon (Ratoc System Engineering, Tokyo, Japan) [12]. In tooth extraction sockets, the bone mass of the sockets (bone fill), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and bone mineral density (BMD) were semi-automatically measured in accordance with the guidelines for assessment of bone microstructures using microCT [13].

2.3. Histomorphometry

Left maxillae were fixed in 10% neutral formalin at euthanasia. The maxillae were demineralized in 10% EDTA, paraffin embedded, and sectioned at 5-μm thickness in serial sagittal sections. Tartrate-resistant acid phosphatase (TRAP) staining was performed to detect osteoclasts on bone surfaces (386A; Sigma-Aldrich). Masson's trichrome staining was conducted for the visualization of collagen fibers (HT15; Sigma-Aldrich). Hematoxylin and eosin (H-E) staining was carried out for the evaluation of soft and hard tissues. Sustained bone exposure without epithelial coverage for > 8 weeks is required to diagnose BRONJ in humans [4]. It requires 2 and > 6 weeks to complete bone remodeling in mice and humans, respectively [14,15]. In this study, osseous and soft tissue wounds with impaired healing and constant bone exposure in the oral cavity for 4 weeks were designated as BRONJ-like lesions, since tooth extraction socket healing in mice is shorter than that in humans. Therefore, the validation of BRONJ-like lesions in this study was histomorphometrically performed by reference to previous reports that validated human BRONJ [16,17] as follows: 1) osteoclast numbers on the bone surface per bone linear perimeter in tooth extraction sockets (N.Oc/BS, #/mm); 2) vital bone area in which there were morphologically normal osteocytes in tooth extraction sockets [living bone (%)]; 3) necrotic bone, defined as the bone area where there are ≥10 adjacent empty or pyknotic osteocyte lacunae that is non-vital [18,19] [necrotic bone (%)]; 4) the number of empty osteocyte lacunae in tooth extraction sockets [empty lacunae ($\#/mm^2$)]; 5) osteocyte numbers of tooth extraction sockets [osteocyte density ($\#/mm^2$)]; 6) collagen fibers quantified in the remaining connective tissue [area of interest (AOI), 200 μm × 500 μm] above the extraction sockets [collagen production (%)]; and 7) infiltration of polymorphonuclear cells (PMN) assessed by quantifying them in the remaining connective tissue within 100 μm of the bone surface (AOI, 100 μm × 500 μm) (PMN infiltration, $\#/mm^2$). It has been reported that higher magnification of H-E-stained sections can revealed the presence of PMNs [20]. Hence, in this study, PMN infiltration was evaluated with H-E stained sections under higher magnification (×400), according to the previous study.

2.4. Immunohistochemistry

To detect blood vessels, the sections were incubated with a CD31 rat anti-mouse primary antibody (ab56299; Abcam, Cambridge, MA, USA) (1:100 dilution) and fluorescent-conjugated rabbit anti-rat Alexa Flour 594 (secondary antibody; Invitrogen, Carlsbad, CA, USA) (1:200 dilution), and mounted using VECTASHIELD Antifade Mounting Medium with DAPI (H-1200 Vector Laboratories, Burlingame, CA, USA). Immune-labeled sections were visualized using immunofluorescent microscopy (Axio Scope A1, Zeiss, Oberkochen, Germany). Blood vessel numbers and areas were quantitatively analyzed by semi-automatically counting the number and area of vessels in the remaining connective tissue above the tooth extraction sockets (AOI, 200 μm × 500 μm) by reference to our previous study [21].

2.5. Statistics

Statistical analyses were blindly conducted. The Shapiro-Wilk test was performed for normality. One-way analysis of variance (ANOVA) for multiple groups was used for parametric data. The Kruskal-Wallis test for multiple groups was used for non-parametric data. All statistical analyses were conducted using Systat 13 (Systat Software, Chicago, IL, USA). An α-level of 0.05 was used for statistical significance. All data are represented as means ± SEM.