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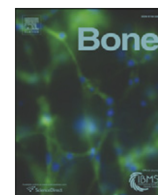

Original Full Length Article

Chemotherapeutic and antiresorptive combination therapy suppressed lymphangiogenesis and induced osteonecrosis of the jaw-like lesions in mice

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ABSTRACT

Osteonecrosis of the jaw (ONJ) is a serious adverse event that occurs predominantly in patients on both antiresorptive and antineoplastic therapies. However, how these combination therapies are connected to the high frequency of ONJ in this particular patient population is unclear. This study's aim was to determine a mechanism of ONJ associated with the combination therapy of antiresorptives and chemotherapeutics. Mice received zoledronic acid (ZA) in conjunction with melphalan or dexamethasone. The maxillary first molars were extracted 3 weeks after the initiation of treatment and wound healing assessed at 4 weeks post-extractions using microcomputed tomography and immunohistochemistry. Mice receiving the combination treatment of ZA and melphalan developed ONJ-like lesions, while ONJ-like lesions were not found in mice on ZA or melphalan monotherapy, or the combination treatment of ZA and dexamethasone. ONJ lesions were characterized by a lack of epithelium, exposed necrotic bone, severe inflammatory cell infiltration, and minimal bone formation. Fluorescent immunohistochemistry showed that lymphatic vessel formation was significantly suppressed in ONJ-like lesions with a concomitant decrease in F4/80^{+} macrophages expressing vascular endothelial growth factor C (VEGFC). Interestingly, significantly suppressed lymphatics were also found in the draining lymph nodes of mice on the combination treatment of ZA and melphalan. Thus, suppressed lymphangiogenesis was strongly associated with the development of ONJ-like lesions in the current study. Since lymphangiogenesis is critical in the resolution of inflammation during wound healing, inflammation control may serve as a potential strategy to prevent ONJ.

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Introduction

Osteonecrosis of the jaw (ONJ) is a devastating condition noted to occur in patients taking antiresorptives. The etiology and pathophysiology of ONJ remain unclear and no established cure currently exists. ONJ predominantly develops in oncology patients with metastatic bone diseases who are on intravenous antiresorptives although it also occurs in patients on oral antiresorptives with much less frequency [1]. These oncology patients have a history of multiple chemotherapy and/or steroid therapies which increase risks of infection, edema, and delayed wound healing [2,3]. Not only cancer patients but also osteoporotic patients with ONJ often have comorbidity, such as asthma and rheumatoid arthritis, which require frequent steroid use [4]. Therefore, the cumulative drug exposure of chemotherapeutics and/or steroids in addition to antiresorptives may have a causative role in the pathophysiology of ONJ [5]. However, mechanisms by which chemotherapeutics and/or steroids contribute to the development of ONJ in patients on antiresorptives are unknown.

ONJ typically develops following tooth extractions but also occurs spontaneously [6]. Spontaneous ONJ may be accompanied with anatomical abnormalities, periodontal diseases, and trauma from daily chewing and oral hygiene practices. It is known that daily oral activity such as chewing, brushing, and flossing transiently induces bacteremia [7,8]. This indicates that a microscopic level disruption of the mechanical barrier (oral mucosa), which is the first line of defense, happens often and results in microbial invasion into the tissue. When the second line of defense, a non-specific immune response, is weakened by myelosuppression via chemotherapy, the risk of microbial invasion into the deeper tissues including the connective tissue and bone greatly increases [9]. This situation manifests as a mucosal injury, a well-known oral complication of chemotherapy [10]. Oral mucosal injuries are often accompanied with viral and/or bacterial infections [11–13]. When an oral mucosal injury involves bone, the infected bone attracts osteoclasts for repair. Although rare, such mucosal injuries result in the development of exposed necrotic bone in the jaw which is collectively defined as chemotherapy-induced ONJ [14–17]. Thus, even without antiresorptive therapy, ONJ could occur in patients on chemotherapy.

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Lymphatic vessels are essential in the resolution of inflammation [18]. Reduced lymphatic vessels lead to persistent edema, a failure in the removal of debris, and inefficient immune responses, leading to impaired wound healing [19,20]. As numerous microorganisms inhabit the oral cavity, a well-developed lymphatic microcirculatory system is found in the oral mucosa to oppose opportunistic bacterial challenges [21]. Since tooth extractions cause mechanical injuries and microbial contamination [22], intense inflammatory and immune responses take place in extraction wounds: osteoclasts resorb the damaged bone and polymorphonuclear leukocytes (PMN) and macrophages initiate to fight bacterial challenges [23,24]. Such inflammatory responses must be resolved for the subsequent tissue formation/regeneration healing process to ensue. As lymphangiogenesis occurs during wound healing to drain soluble antigens and to facilitate immune cell trafficking from wound sites to draining lymph nodes (LNs) [25], lymphangiogenesis and osteoclastic removal of the damaged bone are crucial for tooth extraction wound healing.

In this study we investigated the combination effect of chemotherapeutics and antiresorptives on tooth extraction socket healing in mice to investigate the pathophysiology of ONJ. We found that the combination treatment, but not chemotherapeutic or antiresorptive therapies alone, induced ONJ-like lesions with concomitant suppression of lymphangiogenesis in the tooth extraction wounds and the draining lymph nodes.

Materials and methods

Animals, in vivo injections, and tooth extractions

Experimental Design. VC: mice were injected with 100 μL of saline subcutaneously twice a week. ZA: mice received zoledronic acid subcutaneously at 0.1 mg/kg/w divided into two doses. MEL: mice were intraperitoneally injected melphalan at 7 mg/kg/w. ZA/MEL: mice received the combination treatment of ZA (0.1 mg/kg/w) and MEL (7 mg/kg/w). ZA/DEX: mice received the combination treatment of ZA (0.1 mg/kg/w) and DEX (5 mg/kg/w subcutaneouly divided into 2 doses). Three weeks after the initiation of injections, tooth extractions of the left and right maxillary first molars were performed. Mice were euthanized at 4 weeks post-extractions.

Microcomputed tomography (microCT)

At sacrifice, the left maxillae were dissected, fixed in 10% formalin, and scanned for the extraction sockets at 10 μm voxel resolution with an energy level of 70 kV using microCT (μCT-100, Scanco, Bruttisellen, Switzerland). The sockets were segmented by the semi-manual contouring method and analyzed using built in Scanco software.

Histologic assessments

The right maxillae, draining cervical LNs, and tibiae were dissected, and fixed. The maxillae and tibiae were decalcified in 10% ethylenediaminetetraacetic acid. The LNs and maxillae were processed for cryo-sectioning at 10 μm of serial sagittal sections. The tibiae were paraffin-embedded and sectioned. Hematoxylin and eosin (HE) staining for the LNs, maxillae, and tibiae was performed. Tartrate-resistant acid phosphatase (TRAP) staining for the maxillae and tibiae was conducted to visualize osteoclasts using commercial kits (386A, Sigma-Aldrich) following an adapted protocol [26]. Immunofluorescent staining to visualize lymphatic and blood vessels, macrophages, and vascular endothelial growth factor C (VEGFC) in the LNs and maxillae was performed as follows. Sections were fixed, rehydrated, and subjected to antigen retrieval. Nonspecific protein was blocked. The sections were incubated in a mixture of primary antibodies overnight at 4 °C. Rabbit anti-mouse lymphatic endothelial hyaluronan receptor-1 (LYVE-1) (ab14917, Abcam, Cambridge, MA) and rat anti-mouse CD31 (MCA2388GA, AbD Serotec, Dusseldorf, DE) were used as a pair. The second pair of primary antibodies included rat anti-mouse F4/80 (ab6640, Abcam) and rabbit anti-mouse VEGFC (ab9526, Abcam). Following incubation with the primary antibodies, fluorescent-conjugated secondary antibodies were applied. Alexa Fluor 488 goat anti-rabbit IgG and 594 goat anti-rat IgG (Invitrogen, Carlsbad, CA) at 1:200 were used. DAPI staining (S7113, Millipore, Billerica, MA) was further performed on the sections stained with F4/80 and VEGFC. Stained sections were photomicrographed using a microscopy (BX51, Olympus, Tokyo, Japan) and histomorphometrically analyzed using Image-Pro Plus (Media Cybernetics, Bethesda, MD). The following parameters were assessed: numbers of osteoclasts per linear bone perimeter, osteoblast surfaces, PMN infiltration within 50 μm of bone surfaces, empty osteocyte lacunae numbers in the tooth extraction sockets, bone area within 2 mm of the growth plate in the proximal tibiae, red bone marrow (BM) area in the tibiae, blood vessel numbers, lymphatic vessel numbers, numbers of F4/80$^+$ cells, VEGFC$^+$ cells, and F4/80$^+$VEGFC$^+$ cells, and the size and tissue density of the draining LNs. Necrotic bone in the extraction sockets was defined as the portion of bone in which there were greater than or equal to 10 adjacent empty or pyknotic osteocyte lacunae, since such bone is devital [27,28]. The size and distribution of necrotic bone were histomorphometrically assessed.

Serum and bone marrow RNA

Blood was collected at euthanasia and serum prepared. Serum calcium levels were measured using a commercially available kit (C7503, Pointe Scientific, Canton, MI). BM was isolated from the fresh femurs and red blood cells lysed to prepare mononuclear cells.

Quantitative real-time PCR

Total RNA was extracted from BM cells using the RNAeasy kit (Qiagen, Valencia, CA). First-strand cDNA was synthesized using the SuperScript First-strand system (Invitrogen). Quantitative real-time PCR was performed using an iCycler IQ (BioRad, Hercules, CA) with SYBRGreen mix (Invitrogen). Samples were run in triplicate, and results were normalized to GAPDH and 18S expression.

Western blotting

Isolated BM cells were lysed in RIPA buffer to extract proteins. SDS-PAGE was performed and proteins were blotted to nitrocellulose membranes. Mouse anti-F4/80 (ab6640, Abcam) and goat anti-rat IgG (ab97057, Abcam) were used for a primary and secondary antibodies, respectively. After autoradiography, the blot was reprobed with anti-tubulin (T5168, Sigma-Aldrich). Autoradiographs were digitized and densitometric analysis performed using Image-Pro Plus.

Results

Zoledronic acid/melphalan treatment induced exposed necrotic bone

Healing of tooth extraction sockets was visually examined twice a week. We frequently noticed that large pieces of chows were caught in the extraction wounds which were removed when detected. No mice in the ZA, MEL, and ZA/DEX groups developed ONJ-like lesions. Healing in these treatment groups was grossly similar to that in VC. However, ONJ-like lesions, characterized by a lack of epithelium and exposed necrotic bone, were observed in over 80% (13 out of 16 extraction sockets, 4 sockets were excluded due to root fractures) of the extraction wounds in the ZA/MEL group (Fig. 1A).

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