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Copper accumulation in the sequestrum of medication-related osteonecrosis of the jaw

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ABSTRACT

Bisphosphonates (BPs) have been widely, efficiently, and safely used for the treatment of various bone-related diseases such as osteoporosis. However, concerns about jaw osteonecrosis associated with oral treatment (medication-related osteonecrosis of the jaw [MRONJ]) have been increasing. Although many risk factors for MRONJ have been elucidated, its precise etiology and methods of prevention remain unknown. In this study, we have applied various elemental analysis methods for MRONJ specimens (e.g., X-ray fluorescence with synchrotron radiation [SR-XRF], particle-induced X-ray emission [PIXE], X-ray absorption fine structure [XAFS]) in order to reveal the accumulation and chemical state of trace bone minerals. In four MRONJ sequestra, the characteristic localization of copper (Cu) was observed by SR-XRF. Using micro-PIXE analysis, Cu looked to be localized near the edge of the trabecular bone. The chemical state of the accumulated Cu was estimated using XAFS and the possibility of a Cu–BP complex formation was assumed. Thus, in this study we argue for the feasibility of the trace element analysis to evaluate the potential pathophysiological mechanism of MRONJ.

1. Introduction

As synthetic analogues of pyrophosphate compounds, bisphosphonates (BPs) are selectively uptaken by osteoclasts and strongly inhibit bone resorption by inducing apoptosis (Iwata et al., 2006; Rodan and Fleisch, 1996; Viereck et al., 2002). BPs can be classified into two groups: non-nitrogen containing and nitrogen-containing. Nitrogen-containing BPs, a new-generation of BPs with different action mechanisms than non-nitrogen containing BPs, have been widely used for the treatment of osteoporosis, malignant hypercalcemia, solid cancers inducing bone metastasis, Paget's disease, and multiple myeloma (Russell et al., 2007; Yoneda et al., 2010). However, since Marx et al. in 2003 reported the first case of jaw osteonecrosis in a cancer patient received BPs following dental treatment (Marx, 2003), the reports of medication-related osteonecrosis of the jaw (MRONJ) have been increasing (Bamias et al., 2005). Although many factors related to MRONJ have been identified (Yoneda et al., 2010), such as BP formulation (Marx et al., 2007), local factors (e.g., tooth extraction and dental implants) (Ruggiero et al., 2004; Marx et al., 2005), systemic factors (e.g., anticancer drugs or steroids) (Khamaisi et al., 2007), genetic factors (e.g., presence of P450 CYP2C8) (Sarasquete et al., 2008), and other factors (e.g., drug use, smoking, alcohol) (Matthew and David, 2008; Wessel et al., 2008; Migliorati et al., 2006), the precise risk factors for developing MRONJ are unknown. Although guidelines and position papers suggest systemic management, patient education, antibacterial mouth rinse, oral hygiene maintenance, and BP withdrawal for patients classified as high-risk, there are no clear evidence-based medicine for MRONJ treatment (Yoneda et al., 2010; Ruggiero et al., 2009). Therefore, the establishment of diagnostic markers for MRONJ risk assessment and prevention is urgently needed.

Experimental studies to reveal changes in the chemical components of bone exhibiting MRONJ development have been carried out for serum biomarkers and bone minerals. The relationship between serum biomarkers (e.g., parathyroid hormone (Kim et al., 2013) or C-terminal telopeptide of collagen I (Marx et al., 2007)) and MRONJ development has been studied, although a clear linkage has not been found. Concerning bone minerals, Lowe et al. reported that plasma zinc (Zn) concentration in osteoporosis patients was significantly lower than that in healthy persons, while no significant changes were observed in plasma copper (Cu) concentration (Lowe et al., 2002). Koçer et al.

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reported that BP treatment decreases Zn and Cu in the oral epithelium of rats (Koçer et al., 2013). Therefore, Zn and Cu analysis may be the possible indices to reveal the MRONJ pathophysiological mechanism. However, no literature presents a compelling argument for how to evaluate Zn and Cu levels in MRONJ patients. In addition, those studies discussed the average concentration of Zn and Cu in serum, plasma, and entire tissue. However, those elements would not be homogeneously distributed in bone. Concerning Zn, Gomez et al. demonstrated that it can be detected at the osteon surface and the calcification front (Gomez et al., 1999). Therefore, the quantitative and distribution analyses of Zn and Cu in sequestrum of MRONJ patients might provide useful information to reveal pathophysiology or further risk assessment. However, the concentration of these elements in bone is quite low, so a highly sensitive and position-specific elemental analysis method is required for this purpose. The authors have applied the detection and chemical state analysis methods of metallic trace elements to analyze soft tissues (Sugiyama et al., 2014, 2015; Uo et al., 2015). Synchrotron radiation-induced X-ray fluorescence spectroscopy (SR-XRF) provides information on elements in specimens and X-ray absorption fine structure (XAFS) analysis provides information on the chemical state of target elements. Using these methods, trace metallic elements in biopsy specimens can be detected and analyzed.

In this study, hence, we focus on the distribution and chemical state of trace essential elements contained in bone. SR-XRF and micro-focused particle-induced X-ray emission spectroscopy (micro-PIXE) were applied to an elemental distribution analysis of thin-sectioned specimens of MRONJ sequestrum. The chemical state for concentrated elements in bone was also estimated with an XAFS analysis of the concentrated regions. Subsequently, we estimated the localization of trace mineral accumulation and distribution, and their chemical state in the MRONJ sequestrum.

2. Materials and methods

2.1. Bone specimens

Bone specimens were supplied by Jichi Medical University Hospital, Tochigi, Japan. The samples included four MRONJ-induced sequestrum specimens that were exposed to BP therapy (alendronate [ALN] and zoledronate [ZOL]) and two torus mandibularis specimens that were not exposed to BP therapy. Torus mandibularis is non-neoplastic hyperplasia disease. Histopathologically, laminar bone cortex structure without dysplasia is observed. Therefore, we used torus mandibularis as the control (CON). All patients provided informed consent and the study protocol was approved by the Ethical Committee of Jichi Medical University Hospital (A14-181). Table 1 shows the clinical data for all specimens.

Specimen #1 included mandibular bone tissue from a patient who had received alendronate for osteoporosis. She had been treated with a bridge-type prosthesis eight years earlier, and received BP therapy beginning five years earlier. Direct contact between the two dummy teeth and gingiva and bone exposure in the same region were observed. This patient was diagnosed with MRONJ in light of clinical findings, histopathological findings, and her medical background of receiving BPs.

Specimen #3 included mandibular bone tissue from a patient who had received ZOL for bone metastasis of mammary carcinoma. After her right mandibular molar was extracted, she received BP therapy for a period of two years. Since that time, she experienced strong pain, pus discharge, and bone exposure. This patient was diagnosed with MRONJ using clinical findings, histopathological findings, and medical background.

Specimen #5 included mandibular bone tissue from a patient who had no systemic complications and had not received BPs. He presented to our office with a complaint after bone resection, wherein there was bone prominence in the mandibular lingual region. The denture had pressured onto this area, and the pain and a decubital ulcer were observed. The patient was diagnosed with torus mandibularis using clinical and histopathological findings. The clinical history of the remaining specimens (#2, #4, and #6) is described in "Supplementary material."

All bone specimens were fixed in 4% phosphate-buffered saline–formalin. Decalcification was performed in ethylenediaminetetraacetic acid (EDTA) for three to five days. The specimens were embedded in paraffin and sliced into two thicknesses (4 μm and 8 μm). The 4-μm sample, which was stained with hematoxylin and eosin (H&E), was placed adjacent to the 8-μm sample on Kapton film (12.5 μm thick; Du Pont-Toray Co., Ltd., Tokyo, Japan) and subjected to the following elemental analyses.

2.2. Elemental distribution analysis

2.2.1. Synchrotron radiation X-ray fluorescence spectroscopy (SR-XRF) analysis

XRF analyses of the six specimens were carried out at BL-4A of the Photon Factory at the High Energy Accelerator Research Organization in Tsukuba, Japan. The incident X-ray (12.9 keV) was focused to a 30-μm region using polycapillary optics, and the specimen was irradiated. The specimen stage was scanned in the X–Y plane, two dimensionally, to obtain elemental distribution images. The scanning areas varied within several millimeters, and the scanning steps varied from 40 μm to 100 μm. The obtained XRF data were processed with PyMCA software (Version 4.7.3) after elemental distribution images were obtained.

2.2.2. Preparation of the thin film standard for quantitative analysis

Thin film standard specimens of zinc (Zn) and copper (Cu) were prepared using organometallic compounds and methacrylate resins. First, we prepared the resin monomer matrix: bisphenol A-glycidyl methacrylate (Shin-Nakamura Chemical, Wakayama, Japan) and triethylene glycol dimethacrylate (Tokyo Chemical Industry, Tokyo, Japan) were mixed to a 1:2 weight ratio to be a base monomer. Benzoyl peroxide (Tokyo Chemical Industry, Tokyo, Japan) and camphorquinone (Sigma Aldrich, St. Louis, MO, USA) were added as a polymerization initiator. The acetylacetonates with Zn and Cu (Dojindo, Kumamoto, Japan) were dissolved into the monomer at 0 to 40 ppm. The prepared monomer was spread over the glass plate and polymerized with photo-curing and

Table 1
Clinical data for the patients.

No.	Sex	Age	Diagnosis	BPs	Stage at initial visit	BPs receiving period	Primary disease	Corticosteroid	Site	Trigger dental treatment and oral condition
#1	F	72	MRONJ	ALN p.o.	2	5 years	Osteoporosis	–	Mandibula	Pressure onto the gingiva from two dummy teeth of bridge-type prostheses
#2	F	51	MRONJ	ALN p.o.	2	6 years	Osteoporosis	PSL 5 mg	Mandibula	Tooth extraction
#3	F	65	MRONJ	ZOL i.v.	2	2 years	Bone metastasis of mammary carcinoma	–	Mandibula	Tooth extraction
#4	M	74	MRONJ	ZOL i.v.	2	2 years	Bone metastasis of prostatic carcinoma	–	Maxilla	Chronic apical periodontitis
#5	M	64	Torus mandibularis	–	–	–	–	–	Mandibula	–
#6	F	48	Torus mandibularis	–	–	–	–	–	Mandibula	–

MRONJ—medication-related osteonecrosis of the jaw; F—female; M—male; ALN—alendronate; ZOL—zoledronate; PSL—prednisolone; p.o.— per os; i.v.—intravenous.