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The impact of metastasis on the mineral phase of vertebral bone tissue

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ABSTRACT

The negative impact of metastases on the mechanical performance of vertebral bone is often attributed to reduced bone density and/or compromised architecture. However limited characterization has been done on the impact of metastasis on the mineralization of bone tissue and resulting changes in material behaviour. This study aimed to evaluate the impact of metastasis on micro and nano scale characteristics of the mineral phase of bone, specifically mineral crystal growth, homogeneity of mineralization and changes in intrinsic material properties. Female athymic rats were inoculated with HeLa or Ace-1 cancer cells lines producing osteolytic or mixed (osteolytic & osteoblastic) metastases respectively (N=17 per group). A maximum of 21 days was allowed between inoculation and sacrifice of inoculated rats and healthy age-matched uninoculated controls (N=11). X-ray diffraction was used to assess average crystal size in crushed L1-L3 vertebrae; backscatter electron microscopy and nanoindentation were utilized to evaluate modifications in bone mineral density distribution and material behaviour (tissue hardness and modulus) in sagittal-sectioned, embedded and polished L5 vertebrae. HeLa inoculated samples showed reduced mineral crystal width compared to healthy controls. While both types of metastatic involvement reduced tissue mineral content, pathological osteoblastic bone, specific to Ace-1 inoculated samples, significantly decreased tissue mineral homogeneity, whereas osteolytic bone from HeLa samples saw a slight increase in homogeneity. The modulus and hardness of pathological osteoblastic bone was diminished compared to all other bone. Elucidating changes in material behaviour and mineralization of bone tissue is key to defining bone quality in the presence of metastatic involvement.

1. Introduction

The high incidence (33%) (Wong et al., 1990) and resulting associated morbidity of metastatic involvement in the vertebral bone of cancer patients (Toma et al., 1993) highlights the need to elucidate the impact of tumour presence on bone tissue. While studies have established relationships between metastatic presence on the micro-architecture, bone mineral density and mechanical behaviour of vertebral bone (Kaneko et al., 2004; Wise-Milestone et al., 2012), only minimal attention has been given to the potential modifications to the intrinsic material characteristics of bone tissue and the resulting impact on its material behavioural properties (Nazarian et al., 2008; Richert et al., 2015).

The bone matrix is a composite material consisting of organic and mineral components whose presence, distribution and structure impact the tissue's material properties. While parallel packed collagen- I fibrils provide bone its ductility and toughness, it is the hydroxyapatite mineral phase which is responsible for providing bone with its stiffness and strength. Hydroxyapatite (HA) is a thermodynamically stable form of calcium phosphate, whose crystal structure is hexagonal in nature (Wopenka and Pasteris, 2005). During bone formation, a collagen-I fibril based osteoid is produced from which associated binding matrix proteins such as bone sialoprotein facilitate the nucleation and growth of HA crystal (Hunter and Goldberg, 1993) within the inter and intra-fibrillar space of the collagen fibrils with the c-axis of the hexagonal crystal roughly parallel to long axis of the fibril (Landis, 1996). The primary and secondary nucleation and growth of hydroxyapatite mineral also occurs as a time-dependant process (Preininger et al., 2011) hence there is a variability in the both the size of mineral crystals and the mineral content over time within the bone tissue microenvir-

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onment (Adele, 2003).

Changes in crystal dimension may alter bone's mechanical behaviour. While such changes over time in the average crystal size within bone tissue is natural; pathological bone conditions such as osteoporosis and osteogenesis imperfecta (OI) have been associated with crystal size modification (Thompson et al., 1983; Vetter et al., 1991). Although poor bone quality and mechanical performance is associated with these conditions, the impact on bone crystal size (increased (Thompson et al., 1983) or decreased (Vetter et al., 1991)) is not clear and suggestions have been made as to the importance of mineral size distribution (Fonseca et al., 2014). Metastatic involvement has been shown to decrease mineral crystallinity (Burke et al., 2016b; Bi et al., 2013) (indicative of increased mineral carbonation and/or smaller crystal dimensions) and a feedback relationship may result as mineral crystal size can influence metastatic behavior (Pathi et al., 2011; Zhu et al., 2015).

While modification in bone mineral density (BMD) has been associated with changes in mechanical behaviour and fracture risk, increased emphasis has been placed upon the impact of multiple factors related to bone tissue quality on bone's mechanical behavior (Fonseca et al., 2014; Friedman, 2006; Seeman and Delmas, 2006). Bone mineral density distribution (BMDD) as an assessment of tissue mineralization has been suggested as a potentially important marker of bone quality (Roschger et al., 2007; Roschger et al., 2008) as it provides information on the distribution of mineral within bone tissue (whereas BMD only highlights the average mineral content within an anatomical volume). Trabecular bone is remodelled via the formation of multiple regions (or "pockets") of focal resorption and subsequent bone creation (Clarke, 2008). As bone tissue ages, secondary mineralization causes the amount of mineral in the tissue to increase over time; therefore older bone tissue typically has a higher mineral density than younger bone tissue (Adele, 2003) which can impact its material behavior (Preininger et al., 2011; Tiburtius et al., 2014). The time-dependence of mineral crystal nucleation and growth in conjunction with the constant remodelling of bone via osteolysis (mineral dissolution and osteoid resorption) and ossification, creates a distribution in the mineral content within bone tissue which changes over time (Ruffoni et al., 2007; Ruffoni et al., 2008). However, pathological conditions can potentially affect the rate and frequency of formation of these "pockets" influencing both primary and secondary mineralization, and impacting the distribution of the mineral throughout the bone structure. Bone conditions such as osteoporosis (Roschger et al., 2001), forms of osteogenesis imperfecta (Boyde et al., 1999) and osteomalacia (Roschger et al., 2003) all exhibit changes in bone mineral density distribution.

Modifications within the bone tissue matrix can impact its intrinsic material properties. Several studies have utilized nanoindentation to quantitatively measure the impact of disease, potential treatments or anatomical location on bone's material properties (Maimoun et al., 2012; Hengsberger et al., 2005; Ammann et al., 2007). Pathological conditions have been shown to impact tissue material behaviour. In the case of osteoporosis however, while some research has shown low density bone to have significantly decreased hardness and modulus compared to controls (Maimoun et al., 2012), other studies have not found significant differences in nanoindentation parameters (Guo and Goldstein, 2000; Wang et al., 2008). Metastatic involvement has been shown to reduce the modulus and/or hardness of bone tissue compared to healthy and even osteoporotic samples (Nazarian et al., 2008; Richert et al., 2015).

There is a need to elucidate both elements of bone quality and material behaviour in characterization of the impact of pathology on bone tissue. As such, this study seeks to evaluate the impact of vertebral metastatic involvement on BMDD, mineral crystal size and bone tissue stiffness and hardness. We hypothesize that the influence of metastatic disease on vertebral bone remodelling and osteogenesis will cause modifications in crystal size, mineral distribution and material behaviour properties in vertebral bone.

2. Materials/methods

2.1. Animal model and metastatic inoculation

Animal use protocols, approved by the Ontario Cancer Institute, were adhered to in the utilization of established models of vertebral metastases (Wise-Milestone et al., 2012; Engebraaten and Fodstad, 1999; Won et al., 2010). Athymic female Hsd:RHFoxn1^{rnu} rats (5-6 weeks old) exhibiting pure osteolytic or mixed (osteoblastic + osteolytic) spinal lesions were produced through systemic inoculation with human HeLa cervical cancer cells (previously misidentified as MT-1 cells) or canine ACE-1 prostate cancer respectively (N=17 per group) via intra-cardiac injection. Both cell lines were transfected with the luciferase gene to facilitate semi-quantitative assessment of metastatic growth via bioluminescent imaging (IVIS Imaging system 100, Xenogen, Alameda, California) performed 14 and 21 days post-inoculation. After day 21 imaging, both inoculated and healthy uninoculated age-matched control (N=11) athymic female rats were euthanized via CO2 asphyxiation, their vertebrae harvested and wrapped in saline dampened gauze, and stored in a -20 °C freezer until testing.

2.2. XRD analysis

Methodologies such as x-ray diffraction (XRD) facilitate absolute measurement of average crystal size dimension, helping to characterize observed changes in mineral crystallinity (such as have been shown via Raman Spectroscopy) (Burke et al., 2016b; Bi et al., 2013) within metastatic bone. The first to third lumbar (L1-L3) vertebrae of each rat were isolated and μCT scanned (μCT 100, Scanco, Brüttisellen, Switzerland; Scan Parameters: 55kVp, 200μA, 11W, 7.4 μm resolution) in order to confirm tumour involvement. Visual confirmation of metastasis was demonstrated through features such as voids in the trabecular structure or abnormal bone deposits (Fig. 1). After scanning, tumour burdened vertebrae were isolated and the surrounding soft tissue stripped. The vertebrae were crushed into large fragments, washed with Tris buffer saline solution and dried. Samples were placed in a 2:1 chloroform:methanol solution under agitation for a 24 hour period to facilitate sample defatting. Samples were then placed in 100% methanol solution under agitation for an hour for sample dehydration and chloroform removal, and then dried. The defatted and dried bone samples were then cryogenically impact grinded (Certiprep 6750 Freezer Mill, SPEX SamplePrep, Metuchen, New Jersey) at a rate of 20 impacts per second for 9 minutes to produce a fine bone powder (< 45 μm particle size).

Bone powder samples were scanned utilizing a pXRD instrument (Philips PANalytical XRD system, Amelo, Netherlands. Consists of: PW 1830 HT generator, PW 1050 goniometer, PW3710 control electronics) similar to other studies (Mousny et al., 2008; Ng et al., 2016). In brief, a scan speed of 0.1 °2θ/min was utilized between diffraction angles 24 to 27 °2θ and a scan speed of 0.05 °2θ/min was utilized between diffraction angles 37.0 to 42.0 °2θ in order to analyze the produced 26° and 40° peaks associated with the (002) plane and (310) plane of the HA crystal respectively (Fig. 2). The observed peak broadening (β) was a function of the size/strain of the HA crystals in the samples and the design characteristics of the instrument. This broadening can estimated by the full-width half-maximum (FWHM) of the produced peak. The broadening contribution due to the instrument (β_i) was determined by scanning a silicon wafer reference at the 26 and 47 °2θ diffraction angles and assessing the FWHM of those peaks. The corrected specimen peak broadening ($\beta_{1/2}$) was equal to $\sqrt{\beta^2 - \beta_i^2}$. $\beta_{1/2}$ was then utilized within the Scherrer equation:

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