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Collagen fibril organization within rat vertebral bone modified with metastatic involvement

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ABSTRACT

Metastatic involvement diminishes the mechanical integrity of vertebral bone, however its specific impact on the structural characteristics of a primary constituent of bone tissue, the collagen-I fibril matrix, has not been adequately characterized. Female athymic rats were inoculated with HeLa or Ace-1 cancer cells lines producing osteolytic or mixed (osteolytic & osteoblastic) metastases respectively. A maximum of 21 days was allowed between inoculation and rat sacrifice for vertebrae extraction. Linear polarization-in, polarization-out (PIPO) second harmonic generation (SHG) and transmission electron microscopy (TEM) imaging was utilized to assess the impact of metastatic involvement on collagen fibril organization. Increased observations of deviations in the typical plywood motif or a parallel packing structure and an increased average measured susceptibility ratio (related to relative degree of in-plane vs. out-plane fibrils in the analyzed tissue area) in bone adjacent to metastatic involvement was indicative of change in fibrilar organization compared to healthy controls. In particular, collagen-I fibrils in tumour-induced osteoblastic bone growth showed no adherence to the plywood motif or parallel packing structure seen in healthy lamellar bone, exhibiting a much higher susceptibility ratio and degree of fibril disorder. Negative correlations were established between measured susceptibility ratios and the hardness and modulus of metastatic bone tissue assessed in a previous study. Characterizing modifications in tissue level properties is key in defining bone quality in the presence of metastatic disease and their potential impact on material behaviour.

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1. Introduction

The spread of cancer to bone most commonly affects the vertebral column, occurring in 90% of prostate, 75% of breast, 45% of lung, and 30% of renal terminal cancer patients (Wong et al., 1990). Metastatic spread to the vertebral bodies occurs via the vertebral venous plexus leading cancer cells into a microenvironment within the bone that is rich in growth factors and cytokines conducive to metastatic propagation and growth (Guise, 2010; Ratliff and Cooper, 2004). The presence of tumour cells impacts the natural bone remodelling and turnover cycle causing excess bone formation (osteoblastic), bone resorption (osteolytic) or a mixture of the two. While breast and lung cancers exhibit an affinity to produce osteolytic tumours, prostate cancer yields more osteoblastic disease (Guise, 2010).

Previous studies have highlighted the impact of metastatic disease on the architecture of trabecular bone and its mineral density (Kaneko et al., 2004; Wise-Milestone et al., 2012; Nazarian et al., 2008), however less focus has been placed on potential modifications of tissue microstructure and/or nanostructure that determines the intrinsic material characteristics of the bone matrix. The formation of vertebral bone tissue matrix is completed via endochondral ossification in which a calcified cartilage is used as the base for the formation of an initial woven bone. This woven bone, with its anisotropic collagen type I (collagen-I) fibril foundation, is eventually remodelled to form mature lamellar bone, which consists of a rectilinear array of collagen-I fibrils with hydroxyapatite crystals embedded within the inter and intrafibrillar spacing

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(Shapiro, 2008). The presence, morphology and distribution of these constituents influence the material properties of the bone. Since the hydroxyapatite mineral is critical in providing the strength and stiffness of bone; emphasis is usually placed on the impact of metastases on the mineral content of bone tissue (Kaneko et al., 2004; Wise-Milestone et al., 2012; Nazarian et al., 2008). However, it is the rectilinear array of collagen-I fibrils that provides quasi-brittle bone with its toughness (Wang et al., 2002). Hence, a clear understanding of the potential impact of metastatic involvement on the structure and organization of collagen fibrils is required to characterise potential changes in bone material properties.

Characterization of collagen structure within bone tissue can be performed on microstructural and nanostructural levels. The microstructural level describes the arrangement and organization of fibrils within the tissue. General fibre composite theory highlights the importance of fibre organization and alignment to a material's mechanical performance (Roylance, 2000); which could be applied microscopically to relate the alignment and organization of collagen-I fibrils and the mechanical properties of the bone tissue. This is supported by findings that microstructural changes in collagen fibril arrangement accounted for the maintenance of bone strength despite reduced mineral density in bone subjected to prolonged physical activity (Puustjarvi et al., 1999), and the relationship between orientation dependent fracture energy and collagen orientation in bone (Peterlik et al., 2006). Tissue-level assessed mechanical properties of bone tissue such as modulus and hardness have been shown to be impacted by differences in relative collagen orientation (Zysset et al., 1999; Lau et al., 2010). The nanostructural level describes the morphological and structural characteristics of a single fibril; and interactions between the neighbouring fibrils. Collagen fibril diameter has been previously described as a critical factor related to the biomechanical performance of tissues (Parry et al., 1978; Baek et al., 1998). Modifications in fibril diameter are also often associated with changes in intrafibrillar cross-linking (Christiansen et al., 2000); which in turn is linked to changes in observed bone strength and post-yield properties (Oxlund et al., 1995; Saito et al., 2006; Hernandez et al., 2005).

Our group has previously studied the impact of metastatic presence on the biochemical properties of collagen including hydroxylation and cross-link formation in vertebral bone. Observed changes in intrafibrilar pyridinium cross-link content, non-enzymatic cross-link formation, mature/immature cross-link ratios and hydroxylation of residues show a clear change/modification within collagen fibrils (Burke et al., 2016). Such changes have been linked to modified mechanical behaviour of bone (Saito and Marumo, 2010). However, there is still a lack of information on the impact of metastatic disease on collagen fibril organization, morphology and packing, which also contribute to the mechanical properties of bone (Martin and Boardman, 1993).

Second-harmonic generation (SHG) is a coherent process where two photons of the same wavelength interact with a noncentrosymmetric material and produce a photon of half the wavelength. The intensity and polarization of the SHG signal are highly dependent on the nanostructure and microstructural level of organization of the tissue, and can be used to describe the noncentrosymmetric organization of collagen fibrils (Roth and Freund, 1979; Freund and Deutsch, 1986). Transmission electron microscopy (TEM) can then be used in conjunction with the polarization SHG microscopy to assess the nanostructural details of collagen-I motifs and cross-sections measurements of the dimensions of the collagen-I fibrils in bone and other tissues at high magnifications (Weiner et al., 1997; Rubin et al., 2004; Shauly et al., 1992). These techniques have been utilized to assess the variations in the collagen organization, motif and/or fibril diameter within bone impacted by pathologic conditions such as osteogenia imperfecta and osteoporosis (Kafantari et al., 2000; Nadiarnykh et al., 2007).

This study seeks to assess whether metastatic disease has a significant effect on collagen-I fibril organization and diameter in vertebral bone. The previously established modifications in cross-linking; hydroxylation and other features of collagen-I within bone due to the presence of tumour (Burke et al., 2016) and previously observed changes in fibril orientation and diameter in various bone pathologies (Kafantari et al., 2000; Nadiarnykh et al., 2007) suggest the hypothesis that modifications in both fibril diameter and organization in bone tissue may be present in bone with osteolytic and osteoblastic metastatic involvement.

2. Material/methods

2.1. Animal model and metastatic inoculation

Rat models utilizing systemic inoculation of human HeLa cervical cancer cells and canine Ace-1 prostate cancer cells have been shown to produce osteolytic and mixed osteolytic/osteoblastic metastatic lesions; respectively, within vertebral bone at a success rate of approximately 66% (Wise-Milestone et al., 2012; Engebraaten and Fodstad, 1999; Won et al., 2010). These previously established models were used to simulate the physiologic development of vertebral metastases under an animal use protocol approved by the Ontario Cancer Institute. Athymic female Hsd: RHFoxn1^{rnu} rats (5–6 weeks old) were inoculated with human HeLa cervical cancer cells (previously misidentified as MT-1 cells) to create osteolytic metastases (N = 17). Canine Ace-1 prostate cancer cells were similarly used to produce mixed (osteolytic/osteoblastic) metastases (N = 17). An additional 12 rats were used as healthy controls. The HeLa and ACE-1 cell lines were stably transfected with the luciferase gene to enable bioluminescent image monitoring of tumour growth. Intracardiac injections containing $\sim 1.5 \times 10^6$ cells (in 0.2 mL of media) were conducted under anaesthetic (nose-cone inhalation, 2% isoflurane/air mixture). 21 days after injection, bioluminescence imaging (Xenogen) was performed for detection and semi-qualitative assessment of the degree of metastatic involvement in the spine (Wise-Milestone et al., 2012; Sadikot and Blackwell, 2005). After euthanasia (via CO_2 asphyxiation), vertebrae were harvested, placed in 8% glutaraldehyde fixative solution and maintained at 4 °C. The 11th thoracic (T11) vertebrae were decalcified in 10% EDTA solution and cut in half along the sagittal plane, with one half utilized for polarimetric SHG analysis and the other half for TEM analysis. In cases of premature rat death, the vertebrae were removed immediately and noted accordingly.

2.2. Polarimetric SHG microscopy sample preparation and data acquisition

Linear polarization-in, polarization-out (PIPO) SHG microscopy imaging allows for specific imaging and assessment of collagen fibril organization within tissue. For linear PIPO SHG, T11 half-vertebra samples were dehydrated and paraffin embedded and 5 μm sections were cut and mounted on glass slides. Sections were stained with haematoxylin and eosin (H&E) for histopathologic analysis. Chosen regions of interest (ROI) were divided into trabecular bone that had not completed endochondral ossification (therefore a mixture of lamellar bone, woven bone and cartilage; typically located towards the endplates) versus fully lamellar bone (typically located in the middle of the trabecular centrum). To assess the effect of tumour burden on relative collagen fibril orientation, ROIs were further divided to represent trabecular bone adjacent to tumour and trabecular bone adjacent to unaffected marrow. Additionally, due to the mixed nature of Ace-1 tumour

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