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Mineralization and collagen orientation throughout aging at the vertebral endplate in the human lumbar spine

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ABSTRACT

The human vertebral body and intervertebral disc interface forms the region where the cartilaginous endplate, annulus fibrosis and bone of the vertebral body are connected through an intermediate calcified cartilage layer. While properties of both the vertebral body and components of the disc have been extensively studied, limited quantitative data exists describing the microstructure of the vertebral body-intervertebral disc interface in the spine throughout development and degeneration. Quantitative backscattered scanning electron and second harmonic generation confocal imaging were used to collect quantitative data describing the mineral content and collagen fiber orientation across the interface, respectively. Specimens spanned ages 56 days to 84 years and measurements were taken across the vertebral endplate at the outer annulus, inner annulus and nucleus pulposis. In mature and healthy endplates, collagen fibers span the calcified cartilage layer in all regions, including the endplate adjacent to the central nucleus pulposis. We also observed an abrupt transition from high mineral volume fractions (35–50%) to 0% over short distances measuring 3–15 microns in width across the transition from calcified cartilage to unmineralized cartilage. The alignment of collagen fibers at the outer annulus and thickness of the CC layer indicated that collagen fiber mineralization adjacent to the bone may serve to anchor the soft tissue without a gradual change in material properties. Combining backscattered scanning electron microscopy and second harmonic generation imaging on the same sections thus enable a novel assessment of morphology and properties in both mineralized and soft tissues at the vertebral body-intervertebral disc throughout development and aging.

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1. Introduction

The bone–cartilage, or osteochondral (OC), interface is a region that connects relatively stiff bone (~20 GPa) (Donnelly et al., 2006; Ferguson et al., 2003; Reilly and Burstein, 1975) with relatively soft cartilage (endplate: compressive modulus of 0.4 MPa (Setton et al., 1993); annulus fibrosis: 20 MPa in tension (Acaroglu et al., 1995)). Despite the mismatch in elastic properties between bone and cartilage, compressive, shear and tensile loads are transferred across the junction between the vertebral body and intervertebral disc (VB–IVD) with low incidence of *in vivo* failure (Mow and Huiskes, 2005). The morphology of mineralized tissues at the VB–IVD interface transitions from a thick, fibrous insertion at the outer annulus fibrosis to a thin, porous layer at the cartilaginous endplate (CEP), implying distinct mechanical functions for each distinct anatomical region in the vertebral endplate where soft and mineralized tissues join. Disruption of the endplate structure is commonly seen with aging and degenerative disc disease (Moore, 2006; Roberts et al., 1989, 1996; Thompson et al., 1990). Understanding the structure and properties of the native interfaces between soft and mineralized tissues throughout development and degeneration is a key step to improving spinal implant integration in the context of normal aging and providing information critical to create artificial systems to replace damaged tissue.

At the OC interface, bone and calcified cartilage (CC) are spatially interlocked, while the morphology of the junction between CC and soft cartilage differs depending on the type of cartilage (e.g. hyaline and fibrocartilage) across the vertebral endplate. Across all regions of the vertebral endplate, a CC layer is present throughout development and aging (Nosikova et al., 2012;

Abbreviations: AF, annulus fibrosis; BV/TV, ratio of bone volume to total volume; CC, calcified cartilage; CEP, cartilage endplate; IAF, inner annulus fibrosis; IVD, intervertebral disc; MinVf, mineral volume fraction; NP, nucleus pulposis; OAF, outer annulus fibrosis; OC, osteochondral; qBSE, quantitative backscattered scanning electron; RA, ring apophysis; SCB, subchondral bone; SHG, second harmonic generation; SMI, structural model index; VB, vertebral body; WMGL, weighted mean grey level.

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Roschger et al., 2001; Zizak et al., 2003). The presence of CC across ages and anatomical locations suggests that this mineralized tissue plays an important role for anchoring bone and cartilage and maintaining a robust junction *in vivo*. CC is discernible as a separate tissue from bone at the micron, or tissue (Lakes, 1993; Mow et al., 1992), scale. Studying the CC material at the micrometer length scale provides a basis to describe local changes in mineral content and collagen structure that can influence OC interface behavior with aging. Furthermore, quantitative data on the CC layer, including mineralization level and the underlying collagen orientation across ages and locations, have not been presented previously in the spinal literature.

Second harmonic generation (SHG) imaging utilizes the noncentrosymmetric structure of collagen molecules to image collagen fibers and structures (Stoller et al., 2002). This imaging technique can be used in plastic-embedded tissues and allows visualization of collagen in both mineralized and unmineralized tissue, enabling determination of the collagen orientation both within the CC at the OC interface and immediately adjacent to the CC-cartilage junction. Quantitative backscattered scanning electron microscopy (qBSE) allows quantitation of the spatial distribution of mineral content in tissues based on the atomic density of the material being imaged (Boyde et al., 1995b; Campbell et al., 2012b; Roschger et al., 1998). qBSE is also effective when used on embedded samples and provides a high resolution spatial map of mineral volume fraction (MinVf) in the tissue (Boyde et al., 1995a,b; Campbell et al., 2012b; Ferguson et al., 2003). Multiple studies have shown a relationship between qBSE MinVf with the corresponding mechanical properties of both bone and articular calcified cartilage (Campbell et al., 2012b; Ferguson et al., 2003; Gupta et al., 2005). Thus through qBSE, we are able to relate compositional gradients at the OC interface to mechanical properties. Combining SHG and qBSE on the same area of a sample provides a powerful combination of imaging techniques to quantitatively assess the properties and structure of both mineralized and soft tissues at the OC interface.

This is the first study to combine qBSE and SHG imaging to visualize both the structure of mineralized tissue and collagen fibers in a single region of interest in mineralized tissues. Moreover, this study focuses on the human VB–IVD junction with the goal of elucidating the anchoring mechanisms between bone and cartilage. In order to study the developmental and degenerative changes (at five ages spanning 56 days to 84 years old) at the OC interface, the two main objectives of this paper are to: (i) describe changes in macroscopic endplate morphology across ages and anatomical regions and (ii) quantify micrometer scale organization of the two principal components in mineralized tissues *via* quantitative assessment of mineral volume fraction and collagen fiber orientation. Previous observations have indicated that there is a poor connection between bone and the adjacent, soft cartilage at the vertebral endplate (Inoue, 1981; Roberts et al., 1989), thus a key component of the current study is to explore the microstructure and connectivity of bone, cartilage and CC in this region.

2. Methods

2.1. Samples

Five samples were analyzed ranging in age from 56 days to 84 years old and consisting of: a 56 day old, male (Infant); a 17 year old female (F17); a 30 year old male (M30); a 62 year old female (F62); and, an 84 year old female (F84). Tissue was obtained from two sources: the Musculoskeletal Transplant Foundation, Edison, NJ, USA (Infant, F17) and the University of Colorado School of Medicine (donor cadaver tissue for samples: M30, F62, F84). All tissues were kept frozen at −20 °C until processing. Adult samples (M30, F62 and F84) were from patients with a healthy BMI range of 22.4–24.2. Motion segment samples were collected from the upper lumbar spine L2–L3 disc space (M30, F62, F84); L1–L2 disc space (F17), and the L2 vertebral body (Infant).

Using images taken of the sagittal plane prior to embedding, disc degeneration was graded based on criteria developed by Thompson et al. (Thompson et al., 1990), which describe degenerative changes in each disc component as well as the vertebral body. Disc grades were used to assess overall motion segment health and determine which endplates were a representative of a normal endplate. Disruption of the endplate structure coincided with high degeneration grades (IV–V), and the inferior endplate of F62 and both endplates of F84 were eliminated from CC thickness and collagen orientation quantitation.

2.2. Sample preparation

Samples were hemisected in the mid-sagittal plane on a low speed diamond saw (Isomet, Buehler, Lake Bluff, IL), dehydrated in a series of ethanol solutions to 100%, cleared with acetone, and embedded in poly(methyl methacrylate) (PMMA; Sigma–Aldrich M55909) (Ferguson et al., 2003). The embedding media was then sectioned to expose the mid-sagittal plane and the opposing side was milled to ensure a flat surface for polishing and imaging. All specimens were polished using a series of aluminum oxide pastes (15, 9, 5, 3, 1, 0.1, 0.05 μm) with rayon fine cloths (South Bay Technologies, San Clemente, CA) to a 0.05 micron finish.

2.3. Image analysis

On each endplate, five regions of interest were chosen for image analysis: (1) anterior outer annulus, (2) anterior inner annulus, (3) central nucleus pulposis, (4) posterior inner annulus, and (5) posterior outer annulus (Fig. 1). The inner annulus site was chosen at the ring apophysis (RA) when present because it is a transitional region where the cartilage endplate and annulus fibrosis both join directly to the vertebral body.

2.3.1. Collagen orientation analysis

Prepared samples were imaged with a Zeiss LSM 510 confocal microscope (Carl Zeiss International, Thornwood, NY) at University of Denver Advanced Light Microscopy Core using a 2-photon laser with linear polarization. Samples were excited at 800 nm and the backward propagated photon signal was collected. Using a beam splitter, both the second harmonic signal (400 ± 10 nm) and the 2-photon emission (TPE; 575 ± 125 nm) were recorded. Collagen orientation was quantified using the measurement tool in OrientationJ (Rezakhaniha et al., 2012) with sigma (a signal smoothing function constant for a Gaussian filter) set to 3. The size of each measurement area was kept consistent by overlaying a 225 × 225 micron grid on each image. Rectangles were drawn to analyze the collagen orientation in distinct regions of tissue, *e.g.*, CC, endplate, annulus fibrosis. Collagen orientation versus the distance from the subchondral bone (SCB) was determined by calculating the distance from the coordinates of the center of the measurement region to a line approximating bone position across the image. Coherency, a term which describes the degree to which there is a preferential fiber alignment in the tissue, or the anisotropy of the tissue, was also considered. Coherency, as determined by OrientationJ (Rezakhaniha et al., 2012) is reported on a scale of 0–1, where 0 represents a fully isotropic material and 1 represents a highly aligned anisotropic material. The coherency was always less than 0.02 in regions of images filled only with marrow space (where there are not collagen fibrils). Thus, all orientation

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