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Analysis of the orientation of primary cilia in growth plate cartilage: A mathematical method based on multiphoton microscopical images

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Abstract

The chondrocytic primary cilium has been hypothesized to act as a mechano-sensor, analogously to primary cilium of cells in epithelial tissues. We hypothesize that mechanical inputs during growth, sensed through the primary cilium, result in directed secretion of the extracellular matrix, thereby establishing tissue anisotropy in growth plate cartilage. The cilium, through its orientation in three-dimensional space, is hypothesized to transmit to the chondrocyte the preferential direction for matrix secretion. This paper reports on the application of classical mathematical methods to develop an algorithm that addresses the particular challenges relative to the assessment of the orientation of the primary cilium in growth plate cartilage, based on image analysis of optical sections visualized by multiphoton microscopy. Specimens are prepared by rapid cold precipitation-based fixation to minimize possible artifactual post-mortem alterations of ciliary orientation. The ciliary axoneme is localized by immunocytochemistry with antibody acetylated-α-tubulin. The method is applicable to investigation of ciliary orientation in different zones of the growth plate, under either normal or altered biomechanical environments. The methodology is highly flexible and adaptable to other connective tissues where tissue anisotropy and directed secretion of extracellular matrix components are hypothesized to depend on the tissue's biomechanical environment during development and growth.

Keywords: Cilium; Chondrocyte; Mathematical method; Model; Multiphoton microscopy

1. Introduction

Bone elongation in children occurs through the process of endochondral ossification in cartilaginous growth plates at the ends of long bones. Clonal expansion of stem cells results in columns of chondrocytes whose spatial position within the growth plate mirrors their differentiation stage: cellular proliferation, cellular enlargement (hypertrophy), and cellular apoptotic death followed by replacement of bone on the previously calcified cartilaginous matrix (Fig. 1). The extent of bone elongation achieved depends on the kinetics of chondrocytic activity at each stage of differentiation, and on the rate of regulated transitions between stages. A complex interplay of genetic and epigenetic factors (e.g., endocrine, paracrine, autocrine, nutritional, and biomechanical) influences postnatal longitudinal bone growth, acting primarily at the cellular level through differential effects at specific phases of chondrocytic development and maturation. For recent reviews, see Farnum and Wilsman, 2001, 2002a.

Observed originally in rabbit kidney cells (Zimmerman, 1898), the primary cilium has been suggested to constitute a regular structural feature of virtually all eukaryotic cells within both vertebrates and invertebrates, most characteristically at the incidence of one per cell (for a website on the primary cilium, see: http://members.global2000.net/bowser/cilialist.html). The axonemal structure of primary cilia is characterized by nine doublet microtubules that extend through the axonemal length (Singla and Reiter, 2006). The monocilia of the nodal cells in the embryo show dynein arms which are hypothesized to generate a characteristic propeller-like

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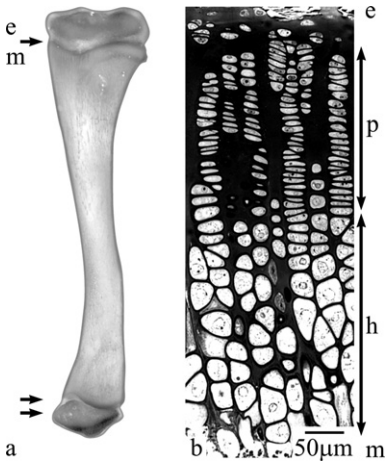

Fig. 1. (a) The proximal (single arrow) and distal (double arrow) growth plates of the tibia of a four-week-old rat are cartilaginous discs, each located between the epiphyseal bone ("e"), and the metaphyseal bone ("m"), at each end of the tibia. (b) This microradiograph shows bone elongation which occurs during the differentiation cascade of chondrocytes in the proximal tibial growth plate of a four-week-old rat. This 1 μm-thick section was stained with methylene blue/azure II to demonstrate the morphology of cells and matrix. The anisotropic arrangement of chondrocytes is demonstrated by the columns of cells, which are a spatial representation of the temporal differentiation of individual chondrocytes. Cellular division is restricted to the proliferative zone ("p"); terminal differentiation is characterized by a significant increase in cellular volume, together with a shape change in the hypertrophic zone ("h"). The death of the terminal chondrocyte occurs just above the metaphyseal bone ("m").

movement (Tabin, 2006; Hirokawa et al., 2006). In general, the primary cilia, that are not nodal, lack dynein arms and are considered to be non-motile in the sense that they lack the ability to generate either a propeller-like movement as for the nodal cilia or a waveform characteristic of the motile cilia whose axonemal core consists of two central microtubules (Bisgrove and Yost, 2006), such as those found in cells of the airway epithelium.. Primary cilia have been observed in the cells of multiple connective tissues including osteoblasts (Tonna and Lampen, 1972); osteocytes (Federman and Nichols, 1974), odontoblasts (Garant et al., 1968), ligament fibroblasts (Bray et al., 2005), meniscal fibroblasts (Le Graverand et al., 2001), periodontal cells (Beertsen et al., 1975), adipocytes (Geerts et al., 1990), and in chondrocytes of articular (Wilsman, 1978) and elastic (Cox and Peacock, 1977) cartilage.

Recent papers recognize primary cilia as sensory organelles for detection and transmission of signals from the extracellular environment to the cell, essential for tissue homeostasis and function (Pazour and Witman, 2003; Whitfield, 2003; Davenport and Yoder, 2005; Schneider et al., 2005; Olsen, 2005). In connective tissues the cilium projects into the extracellular matrix (ECM) and is closely associated with the Golgi apparatus of the cell. Given the highly anisotropic organization of most connective tissues, it has been suggested that the primary cilium may act as a mechanosensor to the local biomechanical environment, and may be significant in the establishment of cellular orientation and directed secretion of ECM components from the Golgi apparatus (Quarmby and Parker, 2005). Poole et al., 1997, 2001 demonstrated that the degree to which the primary cilium extends into the ECM, and whether its axoneme is straight or bent, is variable in articular chondrocytes, and that the configuration of the cilium relative to the chondrocyte changes as fluid flow in the environment changes. Poole has hypothesized that the chondrocytic primary cilium acts as a probe of the ECM and, because of its close association with the Golgi and the microtubule organizing center of the cell, is a key player in establishing cellular shape (Poole et al., 1985, 1997, 2001; Badano et al., 2005). A similar hypothesis has been proposed for the primary cilium in osteoblasts (Wheatley et al., 1996; Quarles, 2005). A hypothesis that the primary cilium is the osteocyte's strain-rate sensing flowmeter unites mechanical and fluid-flow sensory functions (Whitfield, 2003). Attractive as these hypotheses are, they are very difficult to test in the living animal.

If the primary cilium of connective tissue cells is a sensory organelle involved with receiving biomechanical signals that result in directed secretion of the surrounding ECM, one could hypothesize that the orientation of the cilium in three-dimensional space should be consistent with the orientation of the cell itself (i.e. the long axis of the cell on longitudinal sections), or of the orientation of the cells within the tissue (i.e. the long axis of the macroscopic bone). The growth plate is a particularly appropriate connective tissue to investigate this hypothesis since cellular profiles and their orientation have been studied in growth plate cartilage using stereologically based approaches, and it is clear that the long axis of the cell relative to the long axis of the bone changes as chondrocytes progress from proliferation through their terminal differentiation characterized by cellular enlargement during hypertrophy (Farnum et al., 1990; Breur et al., 1991; Hunziker et al., 1987; Buckwalter et al., 1986; Hunziker and Schenk, 1989; Wilsman et al., 1993; Cruz-Orive and Hunziker, 1986).

The purpose of the current study was to develop an experimental technique for analysis of the orientation of the cilium in the growth plate through a new application of mathematical concepts originally developed by Euler to describe the orientation of a segment in a three-dimensional space (Euler, 1755). Adaptation of the classical concepts to the specifics of the biological context is a necessary, important and not obvious step, requiring knowledge of both the mathematical concepts and the biological specifications of the tissue of interest.

The mathematical methodology described in this paper is part of the rapidly growing field of mathematical methods that are developed on images of a given specimen for the purpose of modeling the specimen through the two-step process of data collection from images and consequent computational algorithms on collected data. Angenent et al. (2006) have recently emphasized how this major impetus for new algorithms in signal and image processing has stemmed from the last decade's advent of a variety of faster, more accurate and less invasive imaging devices. All such mathematical algorithms lead to interactive