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Bone mineralization pathways during the rapid growth of embryonic chicken long bones

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ABSTRACT

The uptake and transport of ions from the environment to the site of bone formation is only partially understood and, for the most part, based on disparate observations in different animals. Here we study different aspects of the biomineralization pathways in one system, the rapidly forming long bones of the chicken embryo. We mainly used cryo-fixation and cryo-electron imaging to preserve the often unstable mineral phases in the tissues. We show the presence of surprisingly large amounts of mineral particles located inside membrane-delineated vesicles in the bone forming tissue between the blood vessels and the forming bone surface. Some of these particles are also located inside mitochondrial networks. The surfaces of the forming bones in the extracellular space contain abundant aggregates of amorphous calcium phosphate particles, but these are not enveloped by vesicle membranes. In the bone resorbing region, osteoclasts also contain many particles in both mitochondrial networks and within vesicles. Some of these particles are present also between cells. These observations, together with the previously reported observation that CaP mineral particles inside membranes are present in blood vessels, leads us to the conclusion that important components of the bone mineralization pathways in rapidly forming chicken bone are dense phase mineral particles bound within membranes. It remains to be determined whether these mineral particles are transported to the site of bone formation in the solid state, fluid state or dissolve and re-precipitate.

1. Introduction

Biomineralization pathways refer to the uptake and transport of ions from their source to the site of deposition in a mineralized hard tissue. Many observations have been made on aspects of such pathways in both invertebrates and vertebrates (Weiner and Addadi, 2011). These observations show that different cell types are involved and not only the cells directly responsible for mineralized tissue formation (Akiva et al., 2015; Kerschnitzki et al., 2016; Vidavsky et al., 2014). In some cases dense mineral deposits are formed intracellularly, including within mitochondria (Boonrungsiman et al., 2012; Lehninger, 1970; Mahamid et al., 2011), and the first formed mineral phases often are different from the mature phase found in the mineralized tissue (Akiva et al., 2015; Crane et al., 2006; Mahamid et al., 2010). These observations have been made on diverse biological systems. Here we present a series of observations on the biomineralization pathways of one system, the rapidly growing embryonic long bones of the chicken, in which osteoblasts deposit new bone at the periosteal side, and osteoclasts resorb bone at the endosteal side. This integrated view allows us to simultaneously look into several mineralization pathways, namely ion uptake, deposition and recycling.

The vascular system is known to be intimately associated with bone growth. Bone deposition follows blood vessel formation during primary bone development (Kusumbe et al., 2014; Olsen et al., 2000), and during fracture healing and remodeling (Currey, 2002; Hausman et al., 2001; McKibbin, 1978). One role of the vasculature is to facilitate the transport of the constituents required for bone mineral formation to the new bone deposition site (Kelly, 1983). The amounts of ions that can be transported in solution are limited by the low solubility of bone mineral at physiological pH (McLean and Hinrichs, 1938). Furthermore, the body fluids are supersaturated with respect to calcium and phosphate ions (Holt et al., 1981, 2014; McLean and Hinrichs, 1938), such that there is a real

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danger that the mineral will precipitate. Proteins such as fetuin, matrix Gla-protein and osteopontin are suggested to function in body fluids as inhibitors of ectopic mineral formation (Harmey et al., 2004; Ketteler et al., 2003; Luo et al., 1997; Schinke et al., 1996; Sodek et al., 2000). We recently reported the presence of membrane-bound mineral particles in blood vessels during embryonic chicken long-bone development (Kerschnitzki et al., 2016). The fact that the mineral is stored in the form of dense particles and isolated from the environment by membranes implies that large amounts of mineral can be transported, while minimizing the danger of ectopic vascular calcification. While these membrane-bound mineral particles are not within cells of the blood plasma, they must be formed by cells at some as yet unidentified location. The vascular system, therefore, plays an important role not only for the transport of ions in solution, but also in the distribution of solid mineral towards the embryonic chicken bone.

The observation of membrane-bound solid particles in blood is the starting point for this study. Indeed, it is widely assumed that mineralization of bones involves transport of ions, rather than solid mineral, from their source in the food to the site of deposition in the extracellular matrix (Posner et al., 1978). The notable exception is the so-called matrix vesicles (Anderson et al., 2005). Matrix vesicles bud off from cells, concentrate ions from the extracellular milieu and induce the formation of amorphous calcium phosphate, in the proximity of the forming bone structure (Gay et al., 1978; Golub, 2009). Small particles contained in vesicles inside cells associated with bone formation (presumably osteoblasts) in embryonic mouse bones were shown to be composed of Ca and P, and had a Ca/P ratio of around 0.75 (Mahamid et al., 2011). In osteoblast cultures, Boonrungisman et al. (Boonrungsiman et al., 2012) showed the translocation of similar mineral-containing vesicles from the intracellular environment to the extracellular matrix. Mineral particles were also shown to be present at some distance from the forming bone in the tail fin tissue of larval zebrafish, sometimes in close association with the vasculature (Akiva et al., 2015).

The first mineral phase appearing in the preformed extracellular collagenous matrix of bone is not mature carbonated hydroxyapatite. Crane et al. (Crane et al., 2006) showed that in the forming suture of the mouse calvaria, the predominant mineral phase, presumably located in the preformed matrix, is octacalcium phosphate-like (OCP-like), and amorphous calcium phosphate (ACP)was also possibly present. The presence of OCP-like mineral and different transient disordered phases of calcium phosphate minerals on the surfaces of forming zebrafish tail fin bones was demonstrated by Raman spectroscopy and by X-ray diffraction (Akiva et al., 2015; Bennet et al., 2014; Mahamid et al., 2010). Thus several disparate observations in different bone formation systems point to the importance of intracellular dense mineral deposits in the mineralization pathway.

As embryonic chicken long bones are not only forming but also continuously resorbing bone in distinct locations, we can also study aspects of the resorbing pathway. Osteoclasts dissolve bone mineral by acidifying the underlying bone area and decompose the collagenous bone matrix (Teitelbaum, 2000). Bone resorption products are removed from the resorption site via transcytosis and are eventually released at the basolateral side of the osteoclast (Nesbitt and Horton, 1997; Salo et al., 1997). Thus active osteoclasts must be able to intracellularly store and transport large amounts of resorbed bone mineral. During this process, abundant membrane-bound mineral particles were found to be localized in mitochondrial units (networks) of active osteoclasts (Kawahara et al., 2009; Landis et al., 1977; Lehninger, 1970).

We use the rapidly growing chicken embryo to investigate the mineral deposition pathway and the recycling (resorption) pathway during embryonic chicken long-bone development between the stages E16-E18 (16–18 days post fertilization). During the whole incubation period until hatching after 21 days, the embryo increases many hundredfold in mass (Pines and Hurwitz, 1991; Vleck and Vleck, 1980). In particular from embryonic stage E14 to E19, chicken long-bones roughly double their thickness and the total amount of bone mineral (Yair et al., 2012). Thus at the stage we are studying, bone formation and bone resorption are very rapid.

2. Methods

Since the mineral phases involved in the pathways of bone formation and resorption are highly unstable, it is essential to utilize experimental protocols that prevent the dissolution of these unstable transient mineral structures. Here we use high pressure freezing of freshly dissected bone tissue. Under such rapid freezing conditions the crystallization of water to ice is minimized. This not only avoids rupture of cells and other soft tissues, but also prevents molecular displacements and even chemical reactions from occurring. We examine the bone tissue in a completely hydrated state by utilizing cryogenic scanning electron microscopy (cryo-SEM). In order to obtain 3D information of the distribution of bone mineral during bone formation and resorption, we also use the focused-ion beam (FIB-SEM) in the serial surface view (SSV) mode (Heymann et al., 2006) after application of special plastic embedding protocols to high pressure frozen and freeze substituted samples. These protocols also preserve transient calcium phosphate phases (Kerschnitzki et al., 2016). For further localization and characterization of the mineral phases involved, we use FTIR spectroscopy and synchrotron X-ray fluorescence together with wide angle X-ray diffraction (WAXD).

2.1. Preparation of chick embryos

Fertilized chick eggs were incubated at 38 °C under 80% humidity. Developmental stages are defined according to Hamburger and Hamilton (Hamburger and Hamilton, 1951) and femurs were surgically removed from the animal between stages 42 and 44 (embryonic days post fertilization: E16-E18) after cervical dislocation.

2.2. Micro-CT

Freshly dissected chicken femurs during late embryonic development (E16-E19) were positioned within a home-made plastic holder and placed in the standard sample holder of the micro-CT instrument (XRadia MICRO XCT-400, Zeiss X-ray Microscopy, Pleasanton, CA, USA).The samples were scanned using a voltage source of 40 kV and a current of 200 μA. After recording 1500 projections over 180° the volume was reconstructed with the XRadia software that uses a filtered back projection algorithm. 3D surface rendering was carried out with Drishti – Volume Exploration and Visualization Tool (VizLab, The Australian National University).

2.3. Histology

Chicken femurs were dissected and fixed in 4% paraformaldehyde (PFA)/PBS at 4 °C overnight and decalcified in a solution containing equal parts of 0.5 M EDTA (pH 7.4) and 4% PFA in PBS for 2 days. Then samples were incubated with 0.5 M EDTA (pH 7.4) for 4 days at 4 °C. After fixation and decalcification, tissues were dehydrated to 100% ethanol and embedded in paraffin. The embedded tissues were sectioned to 7 μm thick sections and collected on Fisherbrand Superfrost Plus slides, de-paraffinized and rehydrated to water. Hematoxylin and Eosin (H&E) staining was performed following standard protocols. For osteoclast staining, a leukocyte

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