Histological Aspects on Endochondral Ossification

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Bone is a mineralized tissue composed of calcium phosphates and organic materials such as collagen and proteoglycans. There are two phases of bone mineralization: primary and secondary. Primary mineralization is achieved by osteoblasts. Osteoblasts also produce a large amount of matrix vesicles, which mineralize in nodules (globular assemblies of hydroxyapatite crystals) and then extend into the collagen fibrils secreted by the osteoblasts. In contrast to primary mineralization, secondary mineralization is the process whereby the mineral density of bone increases after primary mineralization. It is postulated that secondary mineralization is regulated through physical crystal maturation, and by the cellular activities of osteocytes embedded in the bone matrix.

Keywords: matrix vesicle ; osteoblast ; bone

1. Introduction

The growth of long bone depends on endochondral ossification, which can be sequentially divided into cartilage matrix mineralization, vascular invasion into the epiphyseal cartilage to expose the mineralized cartilage matrix, osteoblastic migration into the mineralized cartilage cores, and bone deposition to form the primary trabeculae. Hypertrophic chondrocytes play a key role in normal cartilage mineralization, and subsequently in endochondral ossification. These hypertrophic chondrocytes secrete matrix vesicles, extracellular small vesicles that initiate mineralization, and also produce vascular endothelial growth factor (VEGF) allowing the vascular endothelial cells to invade the epiphyseal cartilage. Cartilage mineralization is involved in the modeling of long bones and their changes of shape and size, i.e., the development and growth of the metaphyseal trabeculae. Finely tuned interplays among chondrocytes, vascular endothelial cells, osteoclasts (chondroclasts), and osteoblasts is apparently necessary for adequate endochondral ossification [1].

Histological processes of primary mineralization in the bones can be divided into two phases: matrix vesicle-mediated mineralization and collagen mineralization. In matrix vesicle-mediated mineralization, osteoblasts appear to regulate the secretion speed and the amount of matrix vesicle according to the synthesis of bone matrix. The discovery of matrix vesicles was a breakthrough in the field of bone mineralization [2][3][4][5][6][7][8], and many membrane transporters and enzymes related to matrix vesicle-mediated mineralization have recently been discovered. In addition to matrix vesicle-mediated mineralization, recent reports have suggested that osteocytes putatively regulate the mineralization in the periphery. As osteoblasts and osteocytes are directly connected to each other by means of their cytoplasmic processes, bone mineralization may be regulated by the interplay of osteoblasts and osteocytes. Updated knowledge of the matrix vesicles and osteocytic network in bone mineralization may deepen the understanding of mineral metabolism in bones.

2. Cartilage Mineralization by Hypertrophic Chondrocytes

Epiphyseal cartilage can be divided into three distinctive zones: resting, proliferating, and hypertrophic zones. Chondrocytes form the longitudinal columns in the proliferative and hypertrophic zones, but the proliferative chondrocytes synchronously enlarge in the hypertrophic phenotype [1]. Parathyroid hormone (PTH)-related peptide (PTHrP) has been reported to regulate hypertrophic differentiation of chondrocytes by mediating the Indian hedgehog (IHH)/PTHrP negative feedback [9]. IHH expressed in the prehypertrophic zone (the upper region of the hypertrophic zone) stimulates PTHrP expression in the early differentiation stage of chondrocytes. PTHrP promotes the proliferation activity of chondrocytes by binding to the common receptor of PTH and PTHrP (PTH/PTHrP receptor) in the proliferative zone. PTHrP alternatively inhibits the hypertrophic phenotype of chondrocytes, and IHH expression is then turned off. In addition to IHH/PTHrP negative feedback, another important regulatory factor in chondrocyte proliferation is fibroblast growth factor receptor 3 (FGFR3). Point mutations in FGFR3 cause achondroplasia and thanatophoric dysplasia by continuous activation of the transcription factor Stat1 [10][11]. FGFR3 signaling has also been proposed to increase the pool of proliferating cells by stimulating chondrocytes in the resting zone and promoting their transit to the proliferative zone [12][13]. Thus, the action of

IHH/PTHrP and FGFR3 may be essential for chondrocyte proliferation and differentiation [14]. Hypertrophic chondrocytes have large and translucent cell bodies and produce type I and X collagens, tissue nonspecific alkaline phosphatase (TNAP), proteoglycan, and osteopontin [15][16][17][18][19]. Hypertrophic chondrocytes do not proliferate but acquire mineralization ability in the cartilage matrix. Hypertrophic chondrocytes also reportedly secrete VEGF, an angiogenic molecule that has been implicated in matrix metabolism enabling vascular invasion of the epiphyseal cartilage [20]. Hypertrophic chondrocytes of the epiphyseal cartilage secrete matrix vesicles, in which crystalline calcium phosphates appear, forming hydroxyapatite crystals that grow and eventually break through the membrane to form mineralized nodules in the cartilage matrix. Hypertrophic chondrocytes deposit matrix vesicles in the intercolumnar septae but not in the transverse partitions, consequently forming mineralized longitudinal septae and unmineralized transverse partitions. The regular distribution of mineralized cartilage matrix in the longitudinal intercolumnar septum allows the vertical invasion of vascular endothelial cells, which infiltrate into the cartilage by penetrating the unmineralized transverse partitions. After the formation of these calcified cartilage cores exposed to bone tissues, many osteoblast precursors migrate and attach to the mineralized cartilage cores to deposit abundant organic bone matrices including type I collagen, osteocalcin, osteopontin, and so forth, thereby forming the primary trabeculae. Thus, the process of endochondral ossification involves a well-defined series of events which include the invasion of vascular endothelial cells, osteogenic cell migration, new bone deposition onto the cartilage core, and the formation of primary trabeculae.

3. Vascular Invasion at the Chondro-Osseous Junction

Vascular endothelial cells can invade the epiphyseal cartilage by piercing the incompletely calcified transverse partition of the columns. Researchers demonstrated endomucin-reactive blood vessels invading the chondrocyte lacunae at the chondro-osseous junction [21]. Transmission electron microscopic (TEM) observation verified that the vascular endothelial cells, present in blood vessels close to the cartilaginous matrix, extend their fine cytoplasmic processes into the matrix. The tips of the extended cytoplasmic processes showed fine finger-like structures facing the cartilaginous matrix, suggesting that the apical region of the invading endothelial cells may be partially open. In some observations, cell debris was present inside the blood vessels facing the cartilaginous columns at the chondro-osseous junction, while erythrocytes were found outside the blood vessels. Since the apical region of invading blood vessels might be open, blood vessels could presumably invade the cartilaginous matrix and exclude unnecessary impeditive materials (mainly cellular debris) to avoid accumulation at the junction (**Figure 1**).

Figure 1. Vascular endothelial cells at the chondro-osseous junction. (**a**) Endomucin-immunoreactive (brown color) blood vessels at the chondro-osseous junction under light microscope. (**b**–**f**) TEM images of blood vessels at the chondro-osseous junction. Invading blood vessels are seen beneath the chondrocytic lacunae. (**c**,**d**) When observed under higher magnification as shown in panel c, fine cytoplasmic processes (arrows) are seen extending from the vascular endothelial cell, with invaginations of the cell membranes in the superficial layer of the cartilaginous matrix. (**e**,**f**) Panel e demonstrates cell debris, including erythrocytes from the blood vessels, and panel f reveals an erythrocyte outside the

vessel and cell debris in the vessels. (**g**) Schematic design of vascular invasion at the chondro-osseous junction. HP: hypertrophic chondrocyte; BV: blood vessel, ob: osteoblast. Bar, (**a**) 20 µm, (**b**) 10 µm, (**c**,**e**,**f**) 5 µm, (**d**) 1 µm.

4. Osteoclasts' Function at the Chondro-Osseous Junction

It is well known that osteoclasts, also referred to as chondroclasts, accumulate in the chondro-osseous junction. Osteoclasts at the chondro-osseous junction show intense matrix metalloproteinase (MMP)-9 immunoreactivity [22]. Additionally, MMP-9 immunoreactivity is exhibited in the tips of the vascular endothelial cells facing the cartilaginous matrix, unlike the other areas distant from the chondro-osseous junction [20]. Therefore, osteoclasts and vascular endothelial cells apparently synthesize MMP-9, which dissolves the cartilaginous matrix [23][24]. Vascular invasion rather than osteoclastic resorption seems essential during endochondral ossification. Studies have found that *op/op* mice, *c-fos*$^{-/-}$ mice, and receptor activator of nuclear factor κβ ligand (*Rankl*)$^{-/-}$ mice preserve similar lengths of long bones to those seen in their wild-type counterparts in murine models that lack osteoclasts. However, without osteoclasts, the primary trabeculae form a disorganized but highly connected meshwork in the long bones. As described by Marks and Odgren [25], it seems likely that osteoclastic activity during endochondral ossification resorbs the excess mineralized cartilage matrices and scattered islets of mineralized cartilage in the chondro-osseous junction, enabling the longitudinal arrangement of primary trabeculae. Furthermore, another cell type, septoclasts, also referred to as perivascular cells, may also be involved in vascular invasion during endochondral ossification [26][27][28]. Septoclasts are positive for Dolichos biflorus agglutinin lectin histochemistry [26] and E-FABP [29][30], featuring well-developed Golgi apparatus and several cytoplasmic lysosomes filled with abundant cathepsin B [27]. Researchers speculate that one major function of septoclasts is to remove excess extracellular organic (non-mineralized) debris that would otherwise interrupt the vascular invasion path into the cartilage, and it is unlikely that osteoclasts are designated to resorb the excess mineralized matrices in the cartilage.

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