Matrix Vesicle-Mediated and Osteocytic Regulation of Bone Mineralization

Subjects: Anatomy & Morphology

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Bone mineralization entails two mineralization phases: primary and secondary mineralization. Primary mineralization is achieved when matrix vesicles are secreted by osteoblasts, and thereafter, bone mineral density gradually increases during secondary mineralization. Nearby extracellular phosphate ions (PO_4^{3-}) flow into the vesicles via membrane transporters and enzymes located on the vesicles' membranes, while calcium ions (Ca^{2+}), abundant in the tissue fluid, are also transported into the vesicles. The accumulation of Ca^{2+} and PO_4^{3-} in the matrix vesicles induces crystal nucleation and growth. The calcium phosphate crystals grow radially within the vesicle, penetrate the vesicle's membrane, and continue to grow outside the vesicle, ultimately forming mineralized nodules. The mineralized nodules then attach to collagen fibrils, mineralizing them from the contact sites (i.e., collagen mineralization). Afterward, the bone mineral density gradually increases during the secondary mineralization process.

Keywords: bone mineralization ; osteoblast ; osteocyte ; matrix vesicle

1. Introduction

Bone is mineralized tissue composed of crystalline calcium phosphates and collagen fibrils onto which the calcium phosphate crystals are deposited [1][2][3][4]. The hardness and flexibility of bone, which can provide strength against mechanical force, are derived from calcium phosphates and collagen fibrils, respectively. Osteoblasts secrete a large amount of collagen fibrils, non-collagenous proteins, and proteoglycans, as well as matrix vesicles, into the incompletely mineralized superficial layer of the bone matrix known as the osteoid. A matrix vesicle is a small extracellular vesicle equipped with membrane transporters and enzymes involved in mineralization, such as tissue nonspecific alkaline phosphatase (TNAP) [5][6], ectonucleotide pyrophosphatase/phosphodiesterase (ENPP) [7], sodium-dependent phosphate cotransporter type III (Slc20a1/Pit1 and Slc20a2/Pit2) [8][9][10][11][12], phosphoethanolamine phosphohydrolase 1 (PHOSPHO1) [13][14][15], and ankylosis (ANK) [16][17]. The supply and inflow of calcium ions (Ca^{2+}) and inorganic phosphate ions (PO_4^{3-}) in the initial process of matrix vesicle-mediated mineralization is achieved by the finely-tuned activities of these enzymes and transporters. Hence, matrix vesicle-mediated mineralization is categorized as the primary mineralization that takes place in the osteoid, which then forms a mineralized nodule, also called a calcifying nodule, allowing the collagen mineralization to eventually spread throughout the bone [18][19]. Moreover, non-collagenous proteins and proteoglycans in the osteoid regulate mineralization by modulating the aggregation of collagen fibrils and mineral deposition during primary mineralization [20][21][22][23].

After primary mineralization, the bone mineral density becomes slowly and chronologically elevated in a phenomenon called secondary mineralization, which is independent of osteoblastic bone formation [2]. It is hypothesized that secondary mineralization is achieved by the physicochemical processes of mineral transport in the osteocytic network extended throughout the bone [24]. Therefore, the osteocytes and the meshwork of their cytoplasmic processes appear essential for secondary mineralization involving bone mineral transportation [25][26][27][28]. Osteocytes with well-organized osteocytic lacunar canalicular system (OLCS) sense mechanical stress [29], transport bone minerals, and secrete bone metabolism-regulating molecules [30][31][32][33][34]. It is important that the cytoplasmic processes of osteocytes connect with those of the bone-forming osteoblasts and the narrow channels through which osteocytic processes are opened to the osteoid where the matrix vesicle-mediated mineralization takes place. Therefore, it is postulated that bone minerals, such as Ca^{2+} and PO_4^{3-}, are derived from the activities of TNAP/ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) and PHOSPHO1 inside the matrix vesicles and also from the osteocytic canaliculi that are opened to the osteoid, implicating the interplay of osteoblasts and osteocytes for adequate maintenance of mineralized bone.

2. Matrix Vesicle-Mediated Mineralization

2.1. Nucleation of Calcium Phosphates in Matrix Vesicles

Bone mineralization initiates inside matrix vesicles, which are small extracellular vesicles secreted by osteoblasts [18][19][35][36][37]. Matrix vesicles contain several membrane transporters and enzymes related to mineralization on their plasma membranes and in their interior, thus providing an adequate microenvironment for calcium phosphate nucleation and subsequent growth to eventually form hydroxyapatite crystals [$Ca_{10}(PO_4)_6(OH)_2$]. Regarding calcium phosphate crystal nucleation, it is generally known that Ca^{2+} strongly binds to the negatively charged inner leaflet of the plasma membrane [38]. Plasma membranes consisting of phosphatidylcholine and phosphatidylserine have a substantial capacity for Ca^{2+} binding, with several types of binding sites proposed [39].

2.2. Distribution of Ca and P in the Vicinity of Matrix Vesicles in the Osteoid

Electron energy loss spectroscopy, or EELS, enables elemental mapping and can detect calcium (Ca) and phosphorus (P) at an ultra-structural level [40]. A previous report revealed that Ca and P were highly accumulated inside matrix vesicles; however, Ca was evenly and abundantly distributed in the vicinity of the matrix vesicles, while P was detected predominantly in organic materials, such as collagen fibrils and cells, but not in the matrix vesicle vicinity [40]. Nucleation and growth of calcium phosphate crystals require the influx of Ca^{2+} and PO_4^{3-} inside the matrix vesicles from the extracellular fluid. Taking Ca^{2+} and PO_4^{3-} distribution into consideration, a biological mechanism governing the local synthesis and supplementation of PO_4^{3-}, as well as its subsequent influx into matrix vesicles, must be necessary. In contrast to PO_4^{3-} synthesis and supplementation, Ca^{2+} is abundantly present in the tissue fluid, and annexins, which are acidic phospholipid-dependent Ca^{2+}-binding proteins, are assumed to serve as the Ca^{2+} channels of the matrix vesicles. Annexin A5 is the most abundant protein among annexins [41][42][43], and it appears to display Ca^{2+} channel activity in matrix vesicles.

2.3. Local Synthesis of PO_4^{3-} by the Activities of TNAP and ENPP1

One of the most important enzymes enabling mineralization is TNAP, a glycosylphosphatidylinositol anchor enzyme associated with the cell membranes of matrix vesicles and osteoblastic cells. TNAP can hydrolyze various phosphate esters, especially pyrophosphates (PPi), and is broadly recognized as a hallmark of osteoblastic cells. However, the method of PPi supplementation is important. Currently, it is believed that ENPP1 mainly supplies PPi. ENPP1 is composed of two N-terminal somatomedin B-like domains, a catalytic domain, and a nuclease-like domain. Crystalline structure analysis of ENPP1 demonstrated that the nucleotides are accommodated in a pocket formed by an insertion loop in the catalytic domain of ENPP1, implying a preference for an ATP substrate [7]. Therefore, in bone mineralization, the catalytic activity of ENPP1 may generate PPi, presumably using ATPs in the extracellular fluid. The resultant PPi is then hydrolyzed by TNAP into PO_4^{3-}. However, PPi is also known to inhibit mineralization by binding to nascent hydroxyapatite crystals, thereby preventing crystal overgrowth [44][45][46]. Hence, a balance between PPi and $PO4^{3-}$ is important for normal bone mineralization. TNAP is not uniformly distributed on the cell membranes of osteoblasts; it was distinctly observed on the basolateral sides rather than the secretory (osteoidal) domains [37][47].

2.4. Transport of PPi and PO_4^{3-} via ANK and Pit1/Pit2

ANK, encoded by the progressive ankylosis gene (*Ank*), can serve as a non-enzymatic PPi channel, allowing PPi to pass through the plasma membrane to the outside of the cell [16][17]. As shown in the recent reports, the immunoreactivity of ENPP1 was detected not only in the cell membranes but also in the cytoplasmic region of osteoblasts and osteocytes, indicating the presence of both extracellular and intracellular PPi in these cells [48]. It is therefore likely that the ANK-mediated outflow of intracellular PPi may be involved in the dynamic equilibrium between intra- and extracellular levels of PPi. After the outflow of PPi to the extracellular region, TNAP hydrolyzes PPi into PO_4^{3-}.

Extracellular PO_4^{3-} may pass through the plasma membrane of the matrix vesicles by Pit1 and Pit2 mediation. Pit1 and Pit2 are type III sodium-inorganic phosphate (Pi) co-transporters encoded by Slc20a1 and Slc20a2 [8][9][10][11][12]. Recently, it has been reported that Pit1 and Pit2 form heterodimers, sense extracellular PO_4^{3-} concentrations, and increase the expression of matrix Gla protein (MGP) and osteopontin via the extracellular signal-regulated kinase (ERK) pathway [49][50][51].

2.5. PHOSPHO1 for PO_4^{3-} Production inside Matrix Vesicles

Alternative to the biological function of ENPP1/TNAP, PHOSPHO1 is an enzyme highly expressed in mineralizing osteoblasts and hypertrophic chondrocytes [52]. This enzyme has been implicated in bone and cartilage formation and is

thought to function inside cells and matrix vesicles to generate PO_4^{3-} using phosphocholine and phosphoethanolamine, which are components of the lipid bilayers of matrix vesicles [13][14][15].

3. Development of Mineralized Nodules and Collagen Mineralization

3.1. Growth of Mineralized Nodules

The calcium phosphate crystals that are nucleated inside the matrix vesicles grow in all directions and then penetrate the plasma membrane to exit the vesicles, eventually forming mineralized nodules, which are also referred to as calcifying globules [1][3][4]. Under TEM observation, mineralized nodules appear as globular structures composed of radially assembled hydroxyapatite crystals [53][54]. It seems likely that the growth of mineralized nodules is regulated by non-collagenous proteins in the osteoid. Among these materials, osteopontin is especially suited to regulating mineralization because it is a negatively charged and highly phosphorylated molecule that can effectively inhibit hydroxyapatite formation and growth [6][55]. Osteocalcin is another important bone matrix protein subjected to vitamin K-dependent carboxylation at its glutamate residues.

3.2. Collagen Mineralization

Collagen mineralization begins at the point of contact with mineralized nodules. TEM observations demonstrated that mineralization spreads from the contact point of the mineralized nodules toward the periphery of the collagen fibrils [2]. This finding suggests that collagen mineralization orderly progresses from the contact points with mineralized nodules, presumably allowing the regular deposition of calcium phosphate crystals onto the collagen fibrils. At a higher magnification, the spicules of calcium phosphate crystals can be seen on the fibrillar structures identical to the superhelix (tropocollagen) of collagen fibrils, thus indicating that mineral crystals are deposited on the superhelix, which serves as a scaffold for collagen mineralization. After contact with the mineralized nodules, the collagen fibrils eventually become completely mineralized.

Proteoglycans such as decorin and biglycan, which directly bind the collagen surface through GAG chains, inhibit the growth of mineral crystals [56][57][58]. Collagen mineralization in the osteoid increases proportionally based on the distance from the osteoblasts, whereas the amount of decorin in the osteoid decreases further away from the bone surface [59]. In the osteoid close to the osteoblasts, proteoglycans combined with the surface of newly formed collagen fibrils are localized to the large space between collagen fibrils.

4. Osteocyte Network and the Biological Function of Regulating Bone Mineralization

4.1. Distribution of the Osteocyte Network

Osteoblasts secrete bone matrix proteins and can become embedded in the bone matrix, where they differentiate into osteocytes. Immediately before becoming embedded into the bone matrix, osteoblasts rearrange the actin filament assembly along the cell membranes and the cytoplasmic processes, which resemble that of embedded osteocytes [24]. This implies that the osteoblasts approaching osteocytic differentiation and the newly-differentiated osteocytes decide the geometrical structure of the cellular network of their cytoplasmic processes.

4.2. Osteocyte-Derived Molecules Involved in Peripheral Mineralization

Osteocytes physiologically synthesize several important molecules, e.g., dentin matrix protein (DMP) 1, matrix extracellular phosphoglycoprotein (MEPE), osteopontin, and Phex, for regulating surrounding bone mineralization. DMP1 has a high Ca^{2+}-binding capacity and, therefore, is postulated to play a role in bone mineralization in the vicinity of osteocytes [33]. DMP1 belongs to the small integrin-binding ligand N-linked glycoprotein (SIBLING) family, which also includes MEPE, osteopontin, bone sialoprotein, and dentin sialo-phosphoprotein, and is encoded by a gene located on human chromosome 4q21 and mouse chromosome 5q21 [60][61].

Since osteocytes express abundant MEPE [62], DMP1 [33], and osteopontin, especially in *Hyp* mice fed a high-phosphate diet [63], it can be easily assumed that osteocyte-derived SIBLINGs would regulate peripheral bone mineralization by the osteocytes. This postulation is evidenced by the report that a DMP1 absence results in rickets or osteomalacia in mice [64] and by autosomal recessive hypophosphatemic rickets/osteomalacia (ARHR) in human patients [65]. Hence, osteocytes seem to be involved in the regulation of the surrounding mineralization. However, Phex/SIBLINGs are usually associated

with the congenital deformities rickets and osteomalacia. Therefore, it is necessary to elucidate whether SIBLINGs play an important role in the physiological regulation of bone mineralization in a normal state (**Figure 1**).

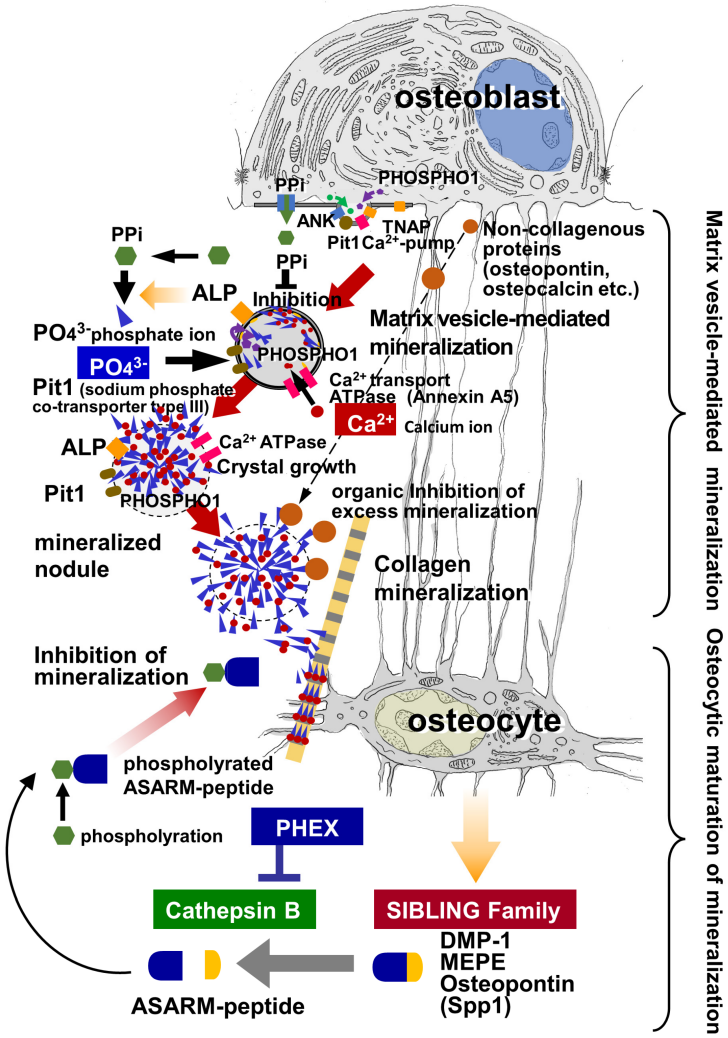

Figure 1. A schematic design of matrix vesicle-mediated mineralization and subsequent osteocytic maturation of mineralization.

5. Cellular Interplay between Osteoblasts and Osteocytes in Bone Mineralization

Osteoblasts secrete matrix vesicles, which provide initiation sites for mineralization during primary mineralization, while osteocytes appear to regulate bone mineralization through Phex/SIBLINGs. Taking these findings into account, the interplay between osteoblasts and osteocytes in the regulation of bone mineralization seems likely. Matrix vesicles secreted by osteoblasts grow into globular assemblies of needle-like calcium phosphate crystals, called mineralized nodules, which then induce collagen mineralization. During nucleation and subsequent growth inside the vesicles, the influx of Ca^{2+} and PO_4^{3-} is promoted by many enzymes and membrane transporters located on the matrix vesicles and mineralized nodules (particularly, they are located on the ruptured membranes of the vesicles).

However, the growth of large, terminal mineralized nodules that are distant from osteoblasts, as well as collagen mineralization, may be regulated by a mechanism other than enzymes associated with matrix vesicles secreted by the osteoblasts. In the osteoid, there seem to be two possible pathways that supply Ca^{2+} and PO_4^{3-} to terminal mineralized nodules and collagen mineralization: one is from the osteoblast-covered bone surface, and the other is from osteocytic canaliculi, which are opened to the osteoid.

6. Conclusions

Primary mineralization in bone is achieved by matrix vesicle-mediated mineralization; matrix vesicles contain a variety of membrane transporters and enzymes involved in the nucleation and subsequent growth of crystalline calcium phosphates inside the vesicles. For proper mineralization, the biological accumulation of Ca^{2+} and PO_4^{3-} in the vesicles is necessary. Of particular importance is the influx of PO_4^{3-} into matrix vesicles, which involves a complex interplay among ENPP1, ANK, TNAP, and Pit1. Crystalline calcium phosphates grow radially, penetrate the vesicle membranes, and then exit the

vesicles to form mineralized nodules, which are globular assemblies of needle-shaped mineral crystals. In contrast to primary mineralization, secondary mineralization increases bone mineral density, presumably due to osteocytic functions. Osteocytes appear to regulate bone mineralization, which is mediated by Phex/SIBLINGs. Thus, bone mineralization is biologically regulated by osteoblasts and osteocytes.

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