Histology of epiphyseal cartilage calcification and endochondral ossification

Norio Amizuka[1], Tomoka Hasegawa[1], Kimimitsu Oda[2], Paulo Henrique Luiz de Freitas[1,3], Kazuto Hoshi[4], Minqi Li[1], Hidehiro Ozawa[5]

[1]Departments of Developmental Biology of Hard Tissue, Graduate School of Dental Medicine, Hokkaido University, Sapporo, Japan, [2]Division of Biochemistry, Graduate School of Medical and Dental Sciences, Niigata University, Niigata, Japan, [3]Oral and Maxillofacial Surgery, Mário Gatti Hospital, Campinas, Brazil, [4]Division of Tissue Engineering, The University of Tokyo Hospital, Tokyo, Japan, [5]Institute for Dental Science, Matsumoto Dental Collage, Shiojiri, Japan

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1. ABSTRACT

Cartilage calcification is carried out by chondrocytes as they hypertrophy and begin to secrete matrix vesicles. Calcification initiates when calcium phosphates appear inside these matrix vesicles, forming hydroxyapatite crystals that eventually break through the membrane to form calcifying globules, as in bone calcification. However, the extracellular environment in cartilage is different from that in bone: cartilage is abundant in proteoglycans but contains a small amount of osteopontin. Hypertrophic chondrocytes secrete vesicles in the cartilaginous matrix of intercolumnar septae only, forming well-calcified longitudinal septae and poorly-calcified transverse partitions. Such pattern of vesicle deposition permits the invasion of endothelial cells, which infiltrate into cartilage and induce migration of osteogenic and osteoclastic cells. Osteoclasts resorb the excess of calcified globules in the partitions, shaping calcified cartilage cores paralleling the longitudinal axis of long bones. After the formation of these calcified cartilage cores, endochondral ossification involves a series of well-defined events in which osteogenic cells deposit new bone onto the cartilage core and form primary trabecules. This review presents the histology of epiphyseal cartilage calcification and endochondral ossification.

2. INTRODUCTION

It is generally accepted that the first clue of the start of a center of ossification in a developing cartilage is hypertrophy of chondrocytes residing in the middle portion of the hyaline cartilage shaft. This finding is consistent with the enlargement of their lacunae at the expense of the intervening cartilaginous matrix. The matrix remaining in the region of the hypertrophic zone becomes calcified. The osteogenic capability of cells from the perichondrium covering the mid-portion of the shaft is subsequently activated. These cells then launch the process of intramembranous ossification. Osteoclasts invade the calcified intramembranous bone inside the mid-shaft cartilaginous anlage, accompanied by migrating blood vessels and osteogenic cells. After that, osteogenic cells differentiate into osteoblasts and start the deposition of new bone in the former hypertrophic zone. The cartilage anlage will then depict cartilaginous extremities sandwiching newly-formed bone, and will be termed epiphyseal cartilage.

The developing epiphyseal cartilage can be histologically divided into three distinct zones: reserve or resting zone, proliferative zone and hypertrophic zone (Figure 1) (1, 2). Chondrocyte precursors differentiate into chondrocytes and start secreting cartilaginous matrix
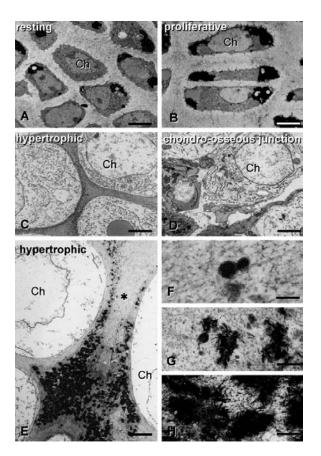
Epiphyseal cartilage calcification

Figure 1. Transmission electron microscopic images of resting, proliferative and hypertrophic chondrocytes.The fetal epiphyseal cartilage can be divided into three distinct zones: reserve or resting zone (A), proliferative zone (B) and hypertrophic zone (C). Chondrocytes (Ch) scattered throughout the resting zone show accumulated glycogen granules and round cell shape (A). Proliferative chondrocytes are aligned in the longitudinal columns known as "stacked coins" (B). Hypertrophic chondrocytes features an enlarged and translucent cell body (C), and sometimes can be seen removed out of their lacunae at the chondro-osseous junction (D). Hypertrophic chondrocytes secrete matrix vesicles from their lateral cell membranes towards the longitudinal intercolumnar septae (an asterisk, E). Highly magnified images of a panel E show matrix vesicles (MV, F) and calcifying globules (CG, G, H) in the hypertrophic zone.Bar, A,B,D: 3µm, C,E: 5µm, F-H: 0.2µm

abundantly, in a process known as appositional growth. Meanwhile, differentiated chondrocytes may undergo cell division and synthesize cartilaginous matrix simultaneously, in what is termed interstitial growth. The normal development and growth of endochondral bone is dependent on a balance between proliferation and differentiation of chondrocytes and the consequent appositional and interstitial growth; it also depends on effective vascular invasion allowing space and nutrition for bone matrix deposition. The spatial progression of chondrocytic differentiation is extremely important for enabling endochondral ossification (Figure 2).

Chondrocytes scattered throughout the resting zone show enlarged cisterns of rough endoplasmic reticulum (rER), accumulated glycogen granules and round cell shape (Figure 1A). They will become proliferative chondrocytes, which align in the longitudinal columns known as "stacked coins" (Figure 1B). Then, proliferative chondrocytes differentiate into the hypertrophic phenotype (Figure 1C), which features an enlarged and translucent cell body and express collagen type I and X, alkaline phosphatase (ALPase), proteoglycans and osteopontin (3-7).

As chondrocytes become hypertrophic, they increase their volume five- to twelve-fold and elevate about three-fold the total amount of extracellular matrix produced (8). Cell organelles in hypertrophic chondrocytes are intact, but scattered throughout the cytoplasm. Glycogen granules, which previously accumulated in the cytoplasm of the resting and proliferative chondrocytes, are dispersed throughout the cytoplasm of hypertrophic cells. Several studies have documented the presence of a transition zone (maturation zone) between the proliferative and hypertrophic zones; in fact, this zone is composed of chondrocytes in the process of differentiation. Hypertrophic chondrocytes cannot proliferate, but acquire the ability of inducing calcification of the cartilaginous matrix. Hypertrophic chondrocytes play a key role in normal cartilage calcification, and subsequently in endochondral ossification, processes that are strictly regulated by a sequence of cellular events.

From a functional viewpoint, hypertrophic chondrocytes secrete matrix vesicles that serve to initiate calcification of the extracellular matrix (9-11). The length of the hypertrophic zone is maintained by a balance between the rate at which chondrocytes enter the hypertrophic phase and the rate at which vascular endothelial cells invade cartilage at the chondro-osseous junction (Figure 2). Hypertrophic chondrocytes are shown to synthesize vascular endothelial growth factor (VEGF), an angiogenic molecule that has been implicated in matrix metabolism and vascular invasion of the epiphyseal cartilage (12).

Cartilage calcification is known to be mediated by the matrix vesicles secreted by hypertrophic chondrocytes. While the molecular mechanism of cartilage calcification is almost same as that seen in bone, the extracellular environment and the cellular events of subsequent endochondral ossification are different from those seen in bone. In this paper, we will review the histological aspects of epiphyseal cartilage calcification and the subsequent endochondral ossification.

2.1. Calcification in hypertrophic zone of cartilage

In cartilage, biological calcification induced by hypertrophic chondrocytes is known to be initiated by small extracellular vesicles referred to as "matrix vesicles" (9-11). Using transmission electron microscopy (TEM), matrix vesicles were shown to accumulate in the longitudinal septa starting from the proliferative zone downwards, featuring "needle-like" mineral crystals in the lower hypertrophic zone (9). We also found small
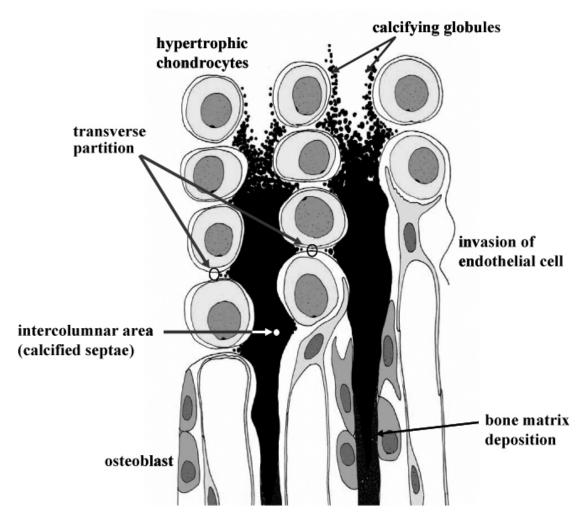
Figure 2. Schematic design for endochondral ossification. Hypertrophic chondrocytes secrete matrix vesicles in the cartilaginous matrix of intercolumnar septae only, forming well-calcified longitudinal septae and poorly-calcified transverse partitions. Endothelial cells infiltrate into cartilage through the transverse partitions, and followed by the subsequent migration of osteogenic cell populations. Hypertrophic chondrocytes are shown to synthesize VEGF, an angiogenic factor that induces vascular invasion of the epiphyseal cartilage.

calcification nodules, *i.e.,* "calcifying globules" as termed by Bonucci (11), and matrix vesicles with mineral crystals starting from the point at which chondrocytes just become hypertrophic, *i.e.,* the maturation zone (Figure 2) (13). Therefore, calcification appears to be initiated by chondrocytes when they begin to be hypertrophic.

Hypertrophic chondrocytes secrete matrix vesicles from their lateral cell membranes towards the longitudinal intercolumnar septae (Figure 1E). In the initial site of cartilage calcification, a line of matrix vesicles and calcifying globules can be seen along the lateral cell membranes of hypertrophic chondrocytes (Figure 1E-H). Consequently, the longitudinal intercolumnar septae undergo proper calcification, and would serve as scaffolds of future primary trabecules on top of which osteoblastic precursors group and start to deposit new bone matrix (Figure 2). In contrast, the transverse partitions of the cartilaginous columns

are incompletely calcified, since hypertrophic chondrocytes do not secrete matrix vesicles abundantly onto this zone. Thus, the well-calcified, intercolumnar longitudinal septae and the incompletely-calcified transverse partition form the calcified cartilage cores for the primary trabeculae, parallel to the longitudinal axis of long bones (Figure 2).

3. MATRIX VESICLE-MEDIATED CALCIFICATION

Discovery of matrix vesicles was a breakthrough for further investigations in the research field of biological calcification (9-11, 14-17). They were first identified in the extracellular matrix of calcifying cartilage. In cartilage, matrix vesicles are limited by a biological membrane sized approximately 30-1000 nm in diameter (9). Hydroxyapatite has a small, ribbon-like structure approximately 25 nm wide, 10 nm high and 50 nm long (18). Calcification starts when hydroxyapatite appears
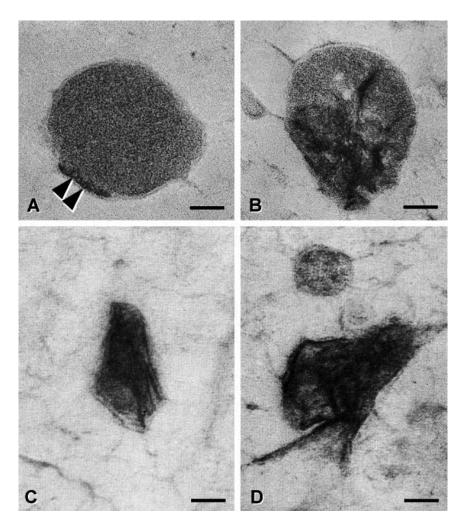
Figure 3. Electron micrographs of matrix vesicle in the rat epiphyseal plate obtained by freeze-substitution methods at liquid helium temperature. Electron dense materials (arrowheads, A) seen along the inner leaflet of the investing membrane of the matrix vesicle (MV) at the early stage of mineralization. Matrix vesicles contain numerous crystal-like structures (arrows B, C). The intravesicular crystal-like structures are exposed to the extravesicular environment, with accumulation and growth of crystals (D). The images were modified from reference (23). Bar, A -D: 50nm

inside these matrix vesicles and progresses as the crystals grow and eventually break through the membrane to form calcifying globules (Figure 3).

Some of these precipitating crystals were initially found associated with the inner leaflet of the matrix vesicle's membrane (See Figure 3A), and it seems likely that crystal nucleation occurs at this specific site. Matrix vesicle membranes are rich in acidic phospholipids such as phosphatidylserine and phosphatidylinositol, which have high affinity for Ca^{2+}. The affinity of phosphatidyleserine to Ca^{2+} is particularly high, and they form a stable calcium phosphate-phospholipid complex (17). The possibility that such complexes may play an important role in crystal nucleation has been pointed out in previous studies (19, 20). Calcium phosphate crystals formed inside matrix vesicles penetrate the plasma membrane, and in the presence of physiological concentrations of extra-vesicular Ca^{2+}, PO_4^{3-} and pyrophosphate (PPi), form the stellate

clusters of "needle-shaped" biological apatite that grow to fill the interstices of the longitudinal septa (21). They are identified as crystalline structures by electron diffraction, but also as non-crystalline structures containing calcium and phosphate by the freeze-substitution method or by cytochemical calcium detection such as κ-pyroantimonate method combined with energy-dispersive X-ray spectroscopy (22-24).

A variety of enzymes and proteins is involved in the metabolism of proteoglycans and pyrophosphate, and are also found in the matrix vesicles. Examples of the former are matrix metalloproteinase (MMP)-3 (25), ALPase (15, 24, 26-29) and ATPase (30). Among the latter are annexin II, V and VI (31), phospholipase A2, carbonic anhydrase II and lactate dehydrogenase (21). These are also believed to regulate crystal precipitation. Even though the plasma membrane of the matrix vesicles would be ripped during the process of crystal growth, calcifying
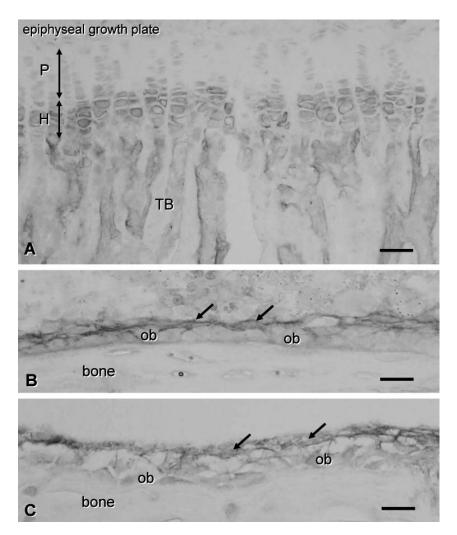
Figure 4. Histochemical localization of ALPase in mouse metaphysis. In epiphyseal growth plate (A), ALPase activity indicated by enzyme histochemistry (red color) is seen along cell membranes of hypertrophic chondrocytes, but not present in the proliferative zone. Observing at higher magnification, both enzyme histochemistry (B) and immunohistochemistry (C) revealed an intense reactivity for ALPase on preosteoblasts (arrows) and on basolateral cell membranes of mature osteoblasts (ob) on the bone surfaces. P: proliferative zone, H: hypertrophic zone, TB: trabecular bone Bar: A: 50μm, B,C: 20μm

globules might not lose these membrane-associated enzymes.

4. FUNCTION OF ALPASE IN CARTILAGE CALCIFICATION

One of the most important enzymes that initiate calcification in the hypertrophic zone of cartilage must be ALPase (Figure 4). ALPase can hydrolyze various phosphate esters, and is responsible for the production of inorganic phosphate: many believe it is a potent inducer of calcification. Recently, evidences based on murine knockout models point to tissue non-specific ALPase essentially as a pyrophosphatase (32). In cartilage, ALPase activity was detected on chondrocytes (3) and matrix vesicles (29, 33). These authors localized the enzyme on the chondrocyte cell membrane and in the matrix vesicles secreted by hypertrophic chondrocytes consistent with our observations (28). Indeed, as shown in Figure 4A, ALPase

activity is seen along the cell membranes of all hypertrophic chondrocytes in the epiphyseal cartilage; proliferative chondrocytes, however, do not express ALPase.

Interestingly, the distribution of ALPase on cell membranes is not uniform in osteoblasts, with showing basolateral and secretory (osteoidal) domains. Takano's group reported that plasma membrane Ca^{2+} transport ATPase was restricted to the osteoidal domain of the osteoblastic cell membrane, while tissue non-specific ALPase was predominantly present on the basolateral domain (34). Consistently, using specific antiserum to tissue non-specific ALPase (35), we could observe relatively intense immunoreactivity and enzymatic activity for ALPase on preosteoblasts and on basolateral cell membranes of mature osteoblasts (Figure 4B,C) (36). Thus, in bone, the membranes featuring intense activity of ALPase are not identical to those that serve as the site of
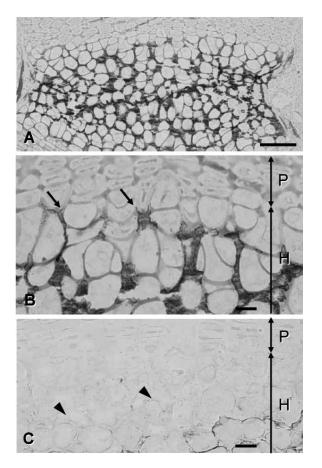
Figure 5. The localization of von Kossa staining and osteopontin in the developing coccygeal vertebare. The upper (A) and middle (B) panels show von Kossa staining in developing coccygeal vertebrae (the middle panel shows a higher image of the upper one). Please note apparent von Kossa staining can be seen in the beginning of the hypertrophic zone (arrows in B), while osteopontin immunoreactivity (brown color) is localized in the middle region of the hypertrophic zone (arrowheads in C). P: proliferative zone, H: hypertrophic zone. Bar: A: 50µm, B,C: 10µm

matrix vesicle formation. In cartilage, however, all the cell membranes of hypertrophic chondrocytes showed uniform and intense ALPase activity. Thus, the distribution pattern of ALPase related to the site of matrix vesicle formation is slightly different in osteoblasts and in hypertrophic chondrocytes.

Mice homogyzous for gene depletion of tissue non-specific ALPase have been generated (37, 38) and mimic severe hypophosphatasia, indicating that ALPase is likely involved in calcification. Tissue non-specific ALPase might act as a pyrophosphatase (32), although other pyrophosphatases may exist. ALPase$^{-/-}$ mice are born with an intact bone, but gradually develop growth retardation as well as skeletal deformities. Although the absence of endogenous tissue non-specific ALPase activity did not result in complete lack of mineral uptake in skeletal

tissues (38), the ALPase$^{-/-}$ mice revealed a severe disturbance of the growth plate, suggesting abnormal endochondral ossification (39). Recently, it was shown that depletion of tissue non-specific ALPase gene results not only in hypocalcification of the skeleton, but also in a severe disorder of mineral crystal alignment in growing long bones with disordered bone matrix architecture (40). More recently, Murshed *et al.* (41) have reported that transgenic mice expressing tissue non-specific ALPase in the dermis showed extracellular calcification consisting of hydroxyapatite crystals. Given the evidence, it seems that ALPase activity is essential for calcification in cartilage and bone, but it is still unknown why the cell membranes with intense ALPase activity in bone are not identical to those forming matrix vesicles.

5. NON-COLLAGENOUS ORGANIC CONSTITUTES IN CARTILAGE CALCIFICATION

5.1. Osteopontin

It seems likely that the calcification process is regulated by a large number of matrix proteins and glycosaminoglycans in cartilage. Among the phosphoproteins, osteopontin is especially suited to the task of regulating calcification, because it effectively inhibits both apatite formation and growth (42, 43). Osteopontin is localized in the periphery of calcifying globules and in the lamina limitans (44-47), where it might act as a blocker of excessive calcification (48). In the calcification process, osteopontin and other organic constituents manifest themselves initially as a protein coating of the crystals, termed "crystal ghosts" by Bonucci (49), and then may coalesce to form crystal globules. *In situ* hybridization demonstrated the expression of osteopontin, as well as osteocalcin and matrix gla protein in the hypertrophic chondrocytes of long bones (4, 50). However, using histochemistry in cartilage, osteopontin was shown to be secreted slightly later than ALPase and formation of matrix vesicles (calcification) in the hypertrophic zone (Figure 5). Consistently, not much osteopontin coated the early calcifying globules in cartilage (Figure 6), which were relatively larger than those in bone (13). Thus, osteopontin appears to be an inhibitor of the growth of calcifying globules.

5.2. Osteocalcin and matrix Gla protein

Osteocalcin and matrix Gla protein are known for containing γ-carboxyglutamic acid and for their ability to bind to mineral crystals (See Figure 7B) (51-53). Recently, the unveiling of the crystal structure of porcine osteocalcin by X-ray analysis revealed a negatively charged protein surface with three Gla residues in helix 1 and conserved Aps residues in helix 2. Such charged surface could bind to Ca^{2+} on a hydroxyapatite crystal lattice (54). Organic components surround the calcifying globules' crystals, forming the so-called crystal ghost (49, 55, 56), where osteocalcin combines with osteopontin (57). It has been reported that calcium-binding organic materials inhibit calcification (43, 58); therefore, all these organic components that potentially bind to hydroxyapatite crystals may act on inhibiting excess accumulation of calcium and phosphate.
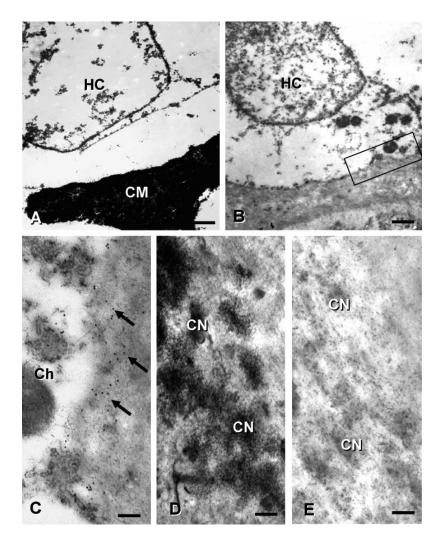
Figure 6. immunolocalization of osteopontin under TEM. In the hypertrophic zone, the cartilage matrix (CM) is well-calcified facing the hypertrophic chondrocyte (HC) (A), and the superficial layer of the cartilage matrix contains gold particles (arrows, C) indicative of osteopontin (B, C). Note panel C is a higher magnification of the square in B. An electron microscopy show calcifying globules (CG) located at the boundary between the proliferative and the hypertrophic zones (D). Immuno-gold particles for osteopontin immunoreactivity are not localized on the electron dense structures identical to the calcifying globules (CG, E). The images were modified from the reference (13). Bar, A,B: 1μm, C-E: 0.2μm

It is interesting that osteocalcin-deficient mice have no obvious skeletal deformity, but showed accelerated bone loss after ovariectomy (59). Boskey *et al* (60) have reported that ovariectomized osteocalcin^{-/-} cortices had lower carbonate/phosphate ratios than did wild-type specimens. Complete inhibition of γ-carboxylated proteins must cause severe skeletal deformities. Warfarin, an inhibitor for γ-carboxylation of glutamine residues, induces an embryopathy consisting of nasal hypoplasia, stippled epiphyses and distal extremity hypoplasia when given to women in the first trimester of pregnancy (61, 62). Administration of warfarin to rats, which decrease the vitamin K-dependent protein of osteocalcin to only 2% of its normal state, resulted in an excessive calcification disorder characterized by complete fusion of the growth plate cartilage of the proximal tibiae, as well as cessation of longitudinal growth (63). In our observations, the administration of warfarin resulted in dispersion of numerous fragments of crystal minerals in osteoid (Figure 7) (64). Therefore, as Hunter *et al* (43) reported, osteocalcin and matrix Gla protein might be involved in the inhibition of nucleation of mineral crystals.

5.3. Proteoglycans

The extracellular matrices, as an ion reservoir, may constitute the adequate microenvironment for the initiation of calcification. Proteoglycans are complexes of various glycosaminoglycan (GAG) chains and core proteins. The GAG chains are highly negatively charged, which can attract free divalent cations such as Ca^{2+} (65, 66). It seems likely that proteoglycans significantly impact the dynamics of the extracellular fluid's mineral ionic content. Since cartilage contains abundant proteoglycans, cartilage matrices may serve as a reservoir for free divalent
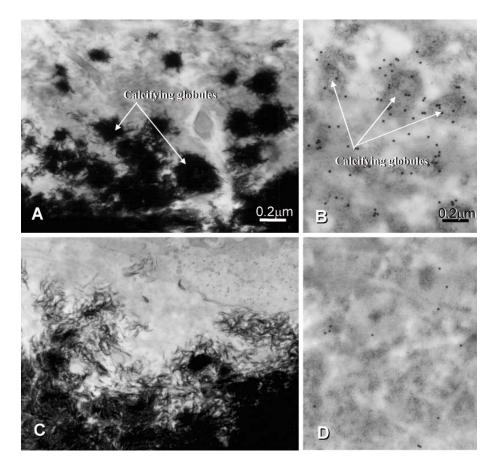
Figure 7. Ultrastructural alterations in calcifying globules and osteocalcin localization. Panels A and C demonstrate the ultrastructure of osteoid from the non-decalcified control and warfarin-administered bones. In control group, the calcifying globules (CG) are composed of mineral crystals featuring fine "needle-like" or "ribbon-like" structures (A). However, the warfarin-administered osteoid shows dispersed needle-like or ribbon-like mineral crystals throughout (C). Osteocalcin immunoreactivity (black particles) can be seen on the calcifying globules (CG, grey globular structures) of the control osteoid (B), while little immunoreactivity for osteocalcin is seen in the warfarin-administered osteoid (D). The images were modified from reference (64). Bar, A -D: 0.2μm

cations. Especially, crystal ghosts appear to be rich in sulfated GAG chains (67, 68), especially, chondroitin sulfate (69).

In cartilage, small leucine-rich proteoglycans -- decorin, biglycan, fibromodulin, lumican, and epiphycan (70, 71), also referred to as PG-Lb (72) -- are present. *In situ* hybridization and immunohistochemistry have verified that hypertrophic chondrocytes in developing epiphyseal growth plate cartilage express high levels of biglycan mRNA (73, 74). Immunoelectron microscopy of the growth plate revealed that the prominent immunolabelling was confined to the Golgi apparatus and cisternae of rER of hypertrophic chondrocytes, and to the early calcified cartilage matrices of the longitudinal septum of the lower hypertrophic zone (74). Therefore, proteoglycans in the extracellular matrix of the lower hypertrophic zone may be degraded by proteases and removed before calcification, and this seems to be the mechanism by which a matrix that does not possess the ability to calcify is transformed into one that has that capability. Interestingly, however, the concentration of sulfate or cartilage proteoglycans was not

shown to change before and after cartilage calcification (6, 75, 76). Decorin/biglycan-double knockout mice revealed osteopenia as a result of impaired GAG-linking to decorin- and biglycan-core proteins, whereas calcification was unaffected (77). Collagen calcification based on the proposed process of removal of small proteoglycans before calcification may deserve further investigation, at least in cartilage.

According to observations of the osteoid in bone derived from the quick frozen-freeze substitution technique with electron energy loss spectroscopy (EELS), which enables elemental mapping at the molecular level, calcium was primarily localized to proteoglycans, whereas phosphate was predominantly localized to collagen fibrils (Figure 8) (28). Therefore, even if the extracellular fluid as a whole is supersaturated with Ca^{2+} and $PO4^{3-}$, it seems feasible that, in non-calcified sites, the extracellular meshwork of organic substances limits the production of hydroxyapatite and inhibits precipitation of calcified crystals by controlling the spatial distribution of Ca^{2+} and $PO4^{3-}$. However, these findings were obtained from
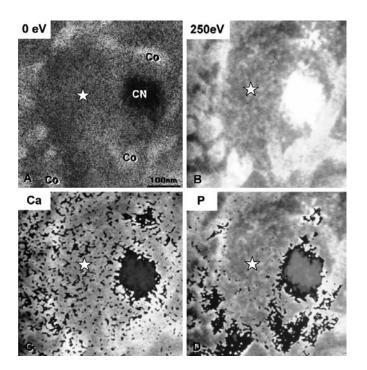
Figure 8. Analysis of the osteoid micromineral environment derived from energy-filtering transmission electron microscopy. A high contrast TEM image was obtained at a 0-eV loss (A). Calcifying nodules (CN) and collagen fibrils (Co) were clearly observed. At a 250-eV loss, the image appeared negative (B). An asterisk indicates the area among calcifying globules and collagen fibrils, probably indicating proteoglycan-rich region. Ca and P are co-localized in calcifying globules. However, the Ca is localized in the area (an asterisk) probably rich in proteoglycan, whereas P often maps to collagen fibril structures. The images were modified from the reference (28). Bar: A-D: 100nm

osteoid in bone, and localization of calcium and phosphate in the cartilage matrix using TEM-EELS is still in progress.

6. INVASION OF ENDOTHELIAL CELLS INTO HYPERTROPHIC ZONE AND OSTEOCLASTIC RESORPTION OF CALCIFIED CARTILAGE MATRIX

There is an abundance of blood vessels and osteoclasts (chondroclasts) where the bottom of hypertrophic zone meets the bone tissue, the so-called erosion zone. The cellular interplay among vascular endothelial cells, osteoclasts and osteoblasts is temporarily and spatially tuned, so that longitudinal primary trabeculae can be formed (Figure 2). In the erosion zone, vascular endothelial cells invade the hypertrophic zone and penetrate the incompletely-calcified transverse partitions, making a way for the migration of osteoclastic and osteoblastic cells. As described, the longitudinal distribution of calcified cartilage matrix seems fundamental for the formation of primary trabecular bone. As vascular invasion progresses, hypertrophic chondrocytes secrete VEGF and guide angiogenesis (Figure 9).

There is another cell-type that may invade the hypertrophic zone, the septoclast (78), which does not belong to the macrophage/osteoclast lineages and is also referred to as perivascular cell (Figure 10) (79). Septoclasts, or perivascular cells, produce cathepsin B abundantly, and thereby, could digest and penetrate into the transverse partition of cartilage matrix at the chondro-osseous junction. The relationship between vascular endothelial cells and septoclasts is, to date, unknown. Septoclasts may aid vascular invasion at the chondro-osseous junction, but further investigation on the cellular function of septoclasts is necessary for clarification of this issue.

What is the osteoclast/chondroclast function in endochondral ossification? Generally, osteoclasts resorb calcified cartilage matrix. Yet, they are consistently found behind invading endothelial cells. Osteoclasts/chondroclasts at the chondro-osseous junction do not resorb the calcified longitudinal septae of cartilage completely, but tend to resorb the excess of calcifying globules in the transverse partitions. This is consistent behavior of bone-resorbing osteoclasts, which remove the superficial layer of calcified bone matrix, but never dig deeply into trabecular bone. We postulate that osteoclasts/chondroclasts could resorb the excessive transverse accumulations of calcifying globules in order to align the calcified cartilage parallel to the longitudinal axis of the long bone, after hypertrophic chondrocytes calcify the longitudinal intercolumnar regions. This supposition is based on the observation that osteoclasts/chondroclasts do not resorb the calcified cartilage matrix completely, so that
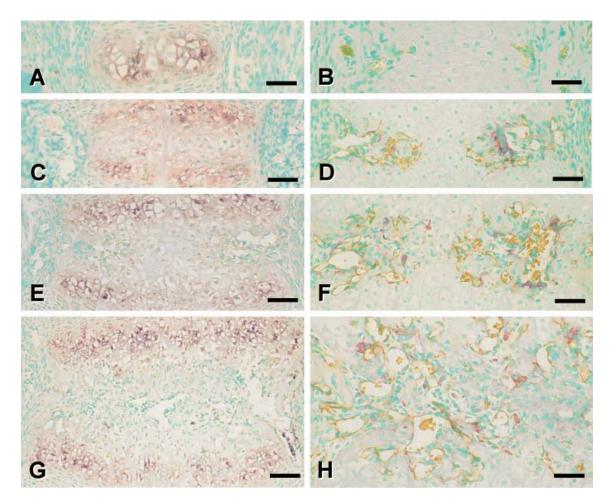
Figure 9. *In situ* hybridization for VEGF mRNA in the developing coccygeal vertebrae, as well as the localization of TRAP-positive osteoclasts and CD31-immunopositive blood vessels. Cartilaginous anlages of coccygeal vertebrae seen in panels A, C, E, G are age-matched to those of B, D, F, H, respectively. Hybridization signals encoding VEGF (violet color) is observed in the hypertrophic zone in all stages of developing cartilage of coccygeal vertebrae (left panels, A, C, E, G). Note invasion of blood vessels (brown color) seems to be going ahead of the migration of TRAP-positive osteoclasts (red) (right panels, B, D, F, H). Bar, A-D: 80µm, E,F: 50µm, G: 120µm, F-H: 30µm

migrating osteoblasts can independently form mixed spicules of cartilage and bone.

Evidence that vascular invasion rather than osteoclastic resorption is dominant for endochondral ossification can be found in osteoclast-absent murine models such as the *op/op* mice, the *c-fos*^{-/-} mice, the receptor activator nuclear factor kappa B ligand (RANKL)[-/-] mice and others. Even without osteoclasts, long bones do grow and elongate; however, there is a picture of mixed spicules with central cartilage cores and bone matrix in the periphery, forming a disorganized meshwork with intense trabecular connectivity.

Osteoclasts at the chondro-osseous junction synthesize more MMP-9 (80) than do osteoclasts located at the end of metaphyseal trabeculae (data not shown). It seems that osteoclasts may recognize the properties of cartilaginous matrix -- proteoglycan-rich or -poor components, calcified or uncalcified -- prior to resorbing it.

For endochondral ossification to happen, osteoclast-driven cartilage resorption may be finely tuned both spatially and temporally. Furthermore, the characteristics of the cartilaginous matrix and the surrounding microenvironment may be important determinants of the process. However, different cellular mechanism on the longitudinal arrangement of primary trabeculae during endochondral ossification may take part in, and therefore, more evidences appears to be necessary for preciously elucidating this issue.

7. APOPTOSIS OF HYPERTROPHIC CHONDROCYTES

It is widely accepted that, under physiological circumstances, chondrocytes in the terminal region of the hypertrophic zone undergo apoptosis as their lacunae are pierced by invading blood vessels (81, 82). Despite their rapid clearance under physiological circumstances, apoptotic chondrocytes have also been identified randomly
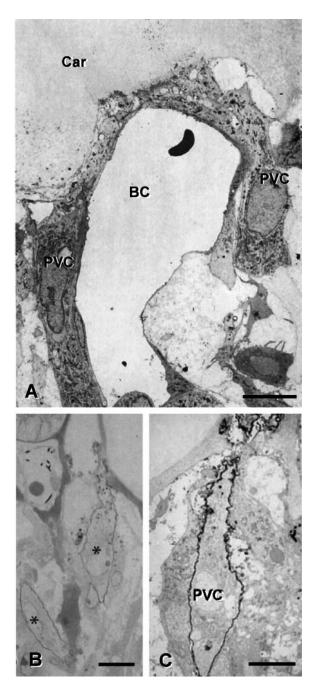
Figure 10. Histological observation of perivascular cell (septoclast) at the chondro-osseous junction. Perivascular cells (PVC) referred to as septoclasts at chondro-osseous junction show fibroblast-like characteristics such as a spindle cell shape and well-developed rough endoplasmic reticulum (A). Lectin histochemistry by Dolichos Biflorus agglutinin (DBA) is detected on the cell membrane of the perivascular cell in the semi-thin section (B) and in electron microscopic image (C). Car: cartilage, BC: blood capillary. The images were modified from reference (79). Bar, A-C: 10μm

throughout the resting and proliferative zones of the epiphyseal cartilage, often in association with viable cells in the same lacuna (83-86). These observations suggest that proliferating chondrocytes can undergo asymmetric cell division and that the surviving cell possesses a competence factor that is not expressed in the cell that dies. Consistently, histochemical investigations have confirmed that chondrocytic death happens through the apoptotic pathway (87).

However, epiphyseal chondrocytes may be able to transdifferentiate into osteoblasts, since terminally differentiated (1, 2) chondrocytes were biosynthetically active. These cells have been shown to synthesize osteogenic markers such as type I collagen, glycosaminoglycans, osteopontin, osteocalcin and osteonectin (4, 5, 88-92). Therefore, we assumed that chondrocytic apoptosis may depend on genetic programming such as Bcl2 expression (93), but may also be influenced by other conditions, *i.e.*, intracellular concentration of Pi (94, 95) and nitric oxide generation (96). We also reported that parathyroid hormone-related peptide deficiency may stimulate chondrocytic apoptosis (97).

In our own observations, there were apoptotic chondrocytes in the hypertrophic zone of adult mice, while 18 day-old mouse fetuses showed intact hypertrophic chondrocytes being removed from the lacunae, as shown in Figure 1D. In contrast, the developing mandibular condyle cartilage at the same fetal age, which unlike appendicular bone has no secondary ossification, showed some apoptotic chondrocytes (98). Taken together, apoptosis of chondrocytes in the hypertrophic zone might be regulated by genetic and environmental stimuli that affect cartilage growth and subsequent endochondral ossification.

8. FUNCTION OF MMP-9, -13 AND MT1-MMP IN ENDOCHONDRAL OSSIFICATION

Endochondral ossification requires proteolytic degradation by means of many proteinases, especially MMPs -9 and -13 (99, 100). These enzymes cleave collagens and aggrecan, two of the main components of cartilaginous matrix. MMP-9, a gelatinase that degrades components of the cartilaginous extracellular matrix with high specificity for degraded collagens, plays a key role in endochondral ossification, specifically capillary invasion into hypertrophic cartilage. In the erosion zone, MMP-9 was shown to be expressed by osteoclastic cells (80, 101), as well as by invading endothelial cells. This means that MMP-9-secreting osteoclasts (chondroclasts) or vascular endothelial cells can digest the incompletely-calcified transverse partitions prior to vascular invasion. However, it is still unknown which cell-type predominantly digests the cartilaginous matrix, and thereby triggers endochondral ossification. Another gelatinase, MMP-3, stromlysin deficiency has not demonstrated altered hypertrophic calcification and subsequent endochondral ossification, so far.

Proteolysis by MMP-13 (collagenase 3) was required for chondrocyte differentiation associated with matrix calcification (102). MMP-13 is expressed in the hypertrophic zone, and is most effective at cleaving type II collagen (103, 104). An inhibitor of MMP-13 was reported to suppress the expression of type X collagen, core binding factor alpha-1 (Cbfal: transcription factor for MMP-13) and MMP-13, and could also hinder calcium incorporation and degradation of type II collagen. However, MMP-13-deficient mice showed no obvious differences in cartilage calcification compared with wild-type littermates, despite the fact that endochondral ossification was dynamically affected (105). It seems that MMP-9 and -13 affect endochondral ossification but do not influence calcification in cartilage.

In contrast, mice homozygous for gene deletion of MT1-MMP (membrane type 1-MMP) referred to as MMP-14 were shown to develop dwarfism, osteopenia, arthritis and connective tissue disease (106). MT1-MMP$^{-/-}$ mice revealed deficient vascularization during secondary ossification of epiphyseal cartilage and growth plate development. Additionally, the authors' group demonstrated that MT1-MMP-dependent dissolution of uncalcified cartilages, coupled with apoptosis of non-hypertrophic chondrocytes, mediates remodeling of these cartilages into other tissues (107). Therefore, MT1-MMP appears to be important for removal of uncalcified cartilage in individual growth.

As shown above, murine knockout models provide a clue for better understanding MMPs in the process of endochondral ossification, and also represent the benchmark for assessing, if not the ultimate physiological role, the dispensability of individual molecules in each envisioned process.

9. CONCLUDING REMARKS

Matrix vesicle-mediated calcification in cartilage, which is achieved by regular cartilage calcification with well-calcified longitudinal intercolumnar septa and poorly-calcified transverse partitions, leads to a series of orchestrated cellular events that result in endochondral ossification. Histological examinations on why hypertrophic chondrocytes secrete matrix vesicles from their lateral cell membranes, what are the functions of septoclasts, and how do proliferative chondrocytes hypertrophy at the same time despite the absence of cellular connections among them may provide clues for better understanding of the mechanisms of calcification in cartilage.

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Send correspondence to: Norio Amizuka, Department of Developmental Biology of Hard Tissue, Graduate School of Dental Medicine, Hokkaido University, Kita 13, Nishi 7, Kita-Ku, Sapporo, Hokkaido, 060-8586, Japan, Tel: 81-11-706-4223, Fax: 81-11-706-4225, E-mail: amizuka@den.hokudai.ac.jp