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What triggers cell-mediated mineralization?

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1. ABSTRACT

Mineralization is an essential requirement for normal skeletal development, but under certain pathological conditions organs like articular cartilage and cardiovascular tissue are prone to unwanted mineralization. Recent findings suggest that the mechanisms regulating skeletal mineralization may be similar to those regulating pathological mineralization. In general, three forms of cell-mediated mineralization are recognized in an organism: intramembranous ossification, endochondral ossification and pathological mineralization. This review summarizes recent work that tried to elucidate how cell-mediated mineralization is initiated and regulated. To explain mineralization, several theories have been proposed. One theory proposes that mineralization is initiated within matrix vesicles (MVs). A second, not mutually exclusive, theory proposes that phosphate induces apoptosis, and that apoptotic bodies nucleate crystals composed of calcium and phosphate. A third theory suggests that mineralization is mediated by certain non-collagenous proteins, which associate with the extracellular matrix. Regardless of the way mineralization is initiated, the organism also actively inhibits mineralization by specific proteins and removal of an inhibitor may also induce mineralization. Although many studies greatly contributed to a better understanding of the mechanisms regulating cell-mediated mineralization, many questions remain about the mechanisms that trigger cell-mediated mineralization and how this process is regulated. Further investigation is necessary to develop in the future novel therapeutic strategies to prevent pathological mineralization.

2. INTRODUCTION

Mineralization is an essential requirement for normal skeletal development, which is generally accomplished through the function of two cell types, osteoblasts and chondrocytes (1). In contrast, soft tissues do not mineralize under normal conditions. However, under certain pathological conditions some tissues like articular cartilage and cardiovascular tissues are prone to mineralization (Table 1) (2;3). Mineralization of articular cartilage contributes to significant morbidity because of its association with joint inflammation and worsening of the progression of osteoarthritis (4). Articular cartilage calcification occurs in association with aging, degenerative joint disease (e.g. osteoarthritis), some genetic disorders and various metabolic disorders (4-7). Similarly, arterial calcification occurs with advanced age, atherosclerosis, metabolic disorders, including end stage renal disease and diabetes mellitus, and some genetic disorders (8). Arterial calcification contributes to hypertension and increased risks of cardiovascular events, leading to morbidity and mortality (9). While pathological mineralization has long been considered to result from a mere physiochemical precipitation of calcium and phosphate, recent studies have provided evidence that soft tissue mineralization is a regulated process, which has many similarities with bone formation (10;11). For now it is unclear why soft tissues have the tendency to mineralize. Therefore, the purpose of this review is to discuss the components involved in cell-mediated mineralization.

3. DIFFERENT TYPES OF MINERALIZATION

3.1. Intramembranous ossification

Clavicles and bones in the regions of the craniofacial skeleton develop by intramembranous ossification, the principle of which is shown in Figure 1, panel 1. During intramembranous ossification, osteoblasts originating from mesenchymal cell condensations are responsible for bone and matrix deposition (12). During this mesenchymal cell condensation, high densities of mesenchymal cells aggregate and start to produce an extracellular matrix (13). Subsequently, mesenchymal cells acquire the typical columnar shape of osteoblasts, increase the synthesis of alkaline phosphatase (APase), and begin to secrete bone matrix, which consists mainly of type I collagen (Figure 1, panel 1A). Osteoblasts than generate phosphate (Pi) by an increase of APase, which is necessary for bone formation (14;15). Numerous ossification centres then develop and eventually fuse (12). Finally, osteoblasts undergo apoptosis (~ 70 %) or terminally differentiate to form osteocytes (~ 30%), which become entrapped in the mineralized bone matrix (14;16;17). Osteocytes are in fact osteoblasts buried within the mineralized bone matrix (Figure 1, panel 1B). Once embedded, they cease their ossification activity and communicate with each other and with cells at the bone surface via a meshwork of cell processes that run through canaliculi in the bone matrix (16). A key regulator of osteoblast differentiation and function is the transcription factor core binding factor alpha 1 (Cbfa1) (12;18-21). Cbfa1 is targeted to the promotors of several bone proteins, such as osteocalcin, bone sialoprotein, APase and type I collagen (18). No bone tissue is formed in Cbfa1 null mice, although a complete cartilaginous skeleton is formed, indicating that Cbfa1 is not essential for chondrogenesis (19).

To maintain bone structure and skeletal growth, remodeling is necessary. The remodeling of bone that has been formed by either intramembranous ossification or endochondral ossification (see below) consists of a strict coupling of bone resorption by osteoclasts and bone formation by osteoblasts that continue throughout life, with a positive balance during growth and with a negative balance during ageing. Osteoclasts are multinucleated giant cells formed from hemopoietic precursors of the monocyte and macrophage series (22). They attach to the bone surface by sealing a resorbing compartment that they acidify by secreting H+ ions. This will than dissolve the bone mineral, and subsequently expose the organic matrix to proteolytic enzymes that degrade it (22).

3.2. Endochondral ossification

Vertebrate long bones form through a process called endochondral ossification, in which a cartilage template produced by chondrocytes, is formed and replaced by bone (Figure 1, panel 2) (12). During the process of endochondral ossification, mesenchymal cell condensation results in differentiation into chondrocytes (13). Chondrocytes then produce a framework of skeletal cartilage template that will subsequently be replaced by bone (12). It has been suggested that this cartilage template in long bones is formed because the mineralized extracellular matrix of bone limits interstitial growth. To achieve rapid and directional growth in an organism, an intermediate structure (cartilage) that can function under high load, and at the same time generate space for new bone formation, would then be very helpful (23). Generally four zones are recognized during endochondral ossification (Figure 1, panel 2A-D). The upper zone is the resting zone (Figure 1, panel 2A), chondrocytes are small and dormant, and mainly type II collagen is produced. Subsequently, in the proliferative zone chondrocytes start to proliferate in vertical columns (Figure 1, panel 2B). The next zone is the hypertrophic zone (Figure 1, panel 2C), where the chondrocytes start to enlarge, produce type X collagen, and increase the synthesis of APase. Finally, hypertrophic chondrocytes induce mineralization (Figure 1, panel 2D), which is correlated with increased levels of Pi in the mineralization zone (24-26). Furthermore, terminally differentiated chondrocytes in the growth plate are deleted from the cartilage by programmed cell death (23). In this final zone, osteoprogenitor cells and hemoatopoietic stem cells arrive via the newly formed blood vessels, which penetrate through the transverse septa of mineralizing hypertrophic chondrocytes. Osteoprogenitor cells beneath the site of vascular invasion differentiate into osteoblasts that aggregate on the surface of the calcified cartilage and deposit bone matrix (osteoid; Figure 1, panel 2E). Bone resorbing osteoclasts start to remodel the newly formed bone (27).

The transcription factor SOX9 is mainly expressed in resting and proliferating chondrocytes, while it is switched off in hypertrophic chondrocytes (28). SOX9 is required for expression of cartilage specific extracellular matrix components, such as collagen types II, IX and XI (12;28). In humans, mutations in SOX9 result in a skeletal malformation syndrome called campomelic dysplasia (29;30). In addition, no chondrocyte specific markers are expressed in SOX9 null cells in mouse chimeras (31). Interestingly, low Cbfa1 expression is detected in hypertrophic chondrocytes, which suggests that hypertrophic chondrocytes may undergo a phenotypic transition towards the osteoblastic phenotype (12). In addition, in Cbfa1 null mice hypertrophic chondrocytes in the femur and humerus are absent (21). The hypothesis that chondrocytes transdifferentiate into osteoblasts is supported by microscopic examinations of cells present at the chondro-osseous junction in the growth plate, which have suggested that indeed chondrocytes differentiate into osteoblasts (32-35). Based on this observation, it is speculated that there are two subpopulations of chondrocytes, one which becomes apoptotic, while the other population trans-differentiates into osteoblasts (32).

3.3. Pathological calcification.

Soft tissues in organisms, like soft tissue structures in joints, skin, heart, blood vessels, kidneys, muscles, lungs, etc. do not mineralize under normal conditions (3). However, as shown in Table 1, under certain pathological conditions some organs mineralize. This is also called pathological mineralization or dystrophic calcification (36). In particular vascular smooth muscle cells in blood vessels are prone to mineralization (Figure 1,

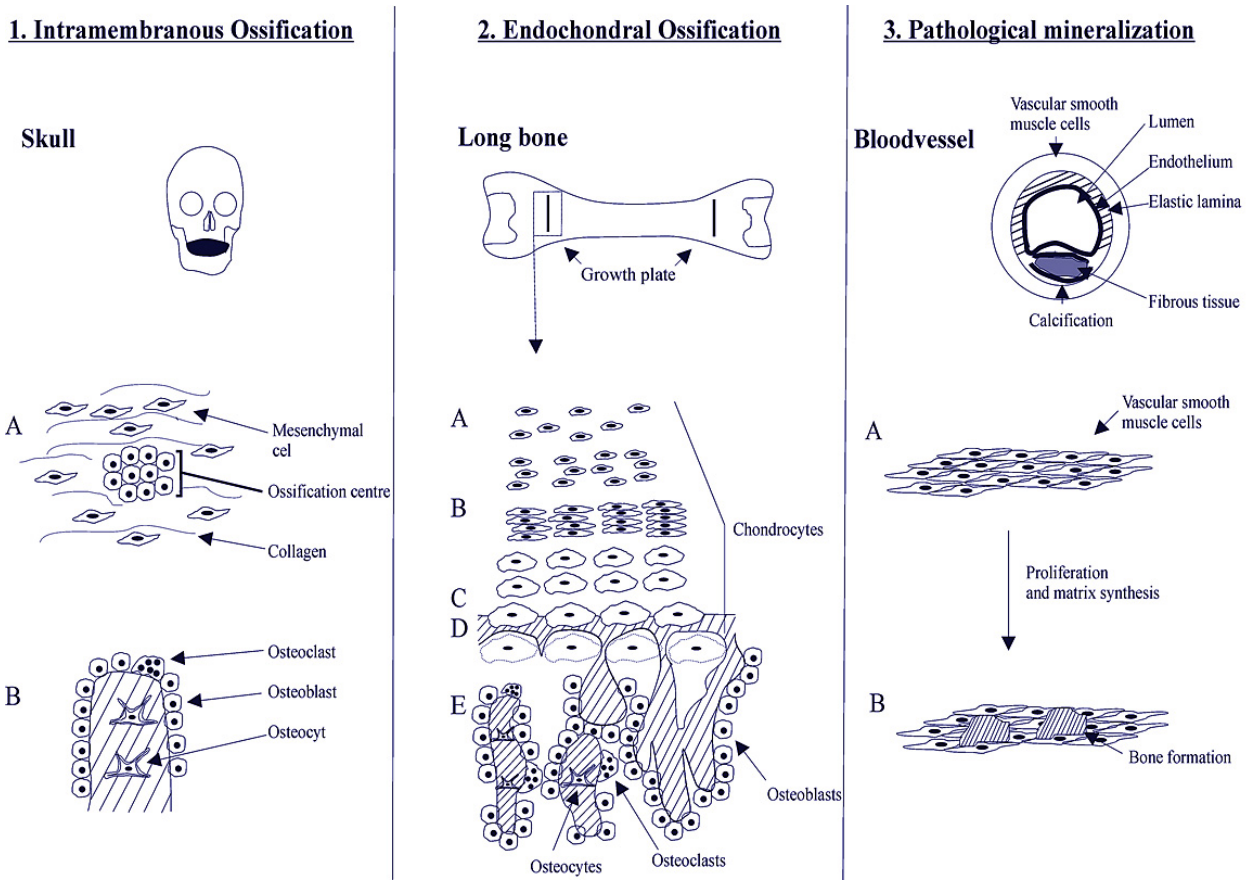

Figure 1. Simplified schematic representation of different forms of cell mediated mineralization. Hatched area represent mineral. Intramembranous ossification. A. Mesenchymal cells aggregate and form nodules (ossification centre), where the cells will differentiate into osteoblasts. B. Bone is formed and remodeled by osteoblasts and osteoclasts. Osteocytes become entrapped in the mineralized matrix. In endochondral ossification 4 zones are recognized in the epiphyseal growth plate. A) resting zone: chondrocytes are small and dormant, mainly type II collagen is produced, B) proliferative zone: chondrocytes start to proliferate in vertical columns, type II collagen is produced, C. Hypertrophic zone: chondrocytes enlarge and start to produce type X collagen, D) Mineralizing zone: hypertrophic chondrocytes start to mineralize, produce matrix vesicles (MVs) and finally die, Type I collagen is now produced E) Osteoblasts grow on the mineralization sheath and produce bone and type I collagen. Some osteoblasts become entrapped in the mineralized matrix and become osteocytes. Osteoclasts remodel newly formed bone. Pathological mineralization (e.g. atherosclerosis). A. Vascular smooth muscle cells in a blood vessel, B. As a result of an atherosclerotic lesion, vascular smooth muscle cells differentiate into osteoblast-like cells and start to induce mineralization.

panel 3A-B). Recent studies have provided evidence that pathological vascular calcification is a regulated process, which has many similarities with bone formation (3;11;37;38). Furthermore, expression of a variety of bone-associated proteins has been found in atherosclerotic plaques (39;40). In addition, it has been reported that 10 to 30 % of the cells in a smooth muscle cell culture system undergo a dramatic phenotypic transition in the presence of relatively high phosphate (> 2 mM). This is characterized by the loss of smooth muscle cell lineage marker expression and upregulation of genes related to the osteogenic phenotype (10;41-43). This suggests that vascular smooth muscle cells undergo phenotypic transition to osteochondroprogenitor-like cells. Moreover, formation of complete bone tissue and bone marrow has been demonstrated in calcified arteries, a phenomenon also called heterotopic (extraosseous) calcification (44;45). In addition, regulated changes in chondrocyte differentiation and viability characteristically seen in growth plate chondrocytes can also occur in mineralizing articular cartilage chondrocytes as a result of osteoarthritis (4).

4. MECHANISM OF CELL-MEDIATED MINERAL DEPOSITION

To induce cell-mediated mineralization, the organism has to create an environment with a local increase of calcium and/or phosphate (Pi), and subsequently organize the nucleation of these ions in an ordered fashion. It has been reported that Pi levels increase considerably from the proliferative to the

Table 1. Forms of pathological mineralization

Organ	Disease	Clinical pattern	Etiology	References
Joints	Tendonitis	Calcification of the tendon	Inflammation, injury, overload	137, 138
	Arthritis	Calcification of articular cartilage	Inflammation, injury, overload, aging	38
	Bursitis	Calcification of bursal walls	Inflammation, overload, aging	139-141
	Bone spurs	Bony projections that grow along the edges of the joints (osteophytes)	Repetitive trauma	142, 143
Heart	Heart valve calcification	Calcification in/around myofibroblasts	Aging, inherited, infection	144
Blood vessels	Atherosclerosis	Calcification in/around vascular smooth muscle cells	Inflammation, injury, aging, high blood pressure	44, 145
Muscles	Myositis ossificans	Bone formation in the muscles	Injury	146
Lung	Metastatic pulmonary calcification	Calcification in alveolar septa	End stage renal disease	147, 148
	Pulmonary ossification	Bone formation in alveolar compartments	Inflammation	148
Skin	Cutaneous ossification	Bone formation in the skin	Injury, inflammation	149
Eyes	Cataracts	Calcification of the lens	Aging	150
Kidney	Kidney stones	Calcium oxalate or calcium phosphate stones	Inherited, mineral imbalance	151
Brain	Bilateral striopallidodentate calcinosis	Symmetric calcification of the basal ganglia	Aging, inherited, infection	152, 153
Spinal cord	Neurogenic heterotopic ossification	Bone formation in spinal cord	Injury	154
Tumor	Osteosarcoma	Bone cancer that may metastasize elsewhere	Unknown	155

hypertrophic zone in the growth plate (25;26). Furthermore, patients with end-stage renal disease (ESRD) develop vascular calcification, which is correlated with an increased serum Pi concentration that typically exceeds 2.0 mM (normal level: 1.4 mM) (10;46). In addition, APase, which cleaves phospho-compounds to inorganic phosphate, is highly increased in mineralization competent cells like osteoblasts, hypertrophic chondrocytes and mineralizing vascular smooth muscle cells. The role of APase in mineralization is essential, since APase deficient mice show impaired skeletal mineralization (47). However, the identity of the physiological organic Pi substrate is not defined.

There is much debate regarding the biochemical mechanisms that initiate mineralization subsequent to the increase in calcium and/or Pi. Figure 2 summarizes the principal factors that are believed to play a role in the process of tissue mineralization. One theory proposes that mineralization is initiated within matrix vesicles (MVs) (48). A second, not mutually exclusive, theory proposes that Pi induces apoptosis, and that apoptotic bodies nucleate crystals composed of calcium and Pi (23;49). A third theory suggests that mineralization is mediated by certain non-collagenous proteins (NCPs), which associate with the extracellular matrix (50-52). Organisms also actively inhibit mineralization by secreting specific proteins and removal of an inhibitor may induce mineralization as well (Figure 2) (53).

4.1. The role of matrix vesicles

It has been hypothesized that cell-mediated mineralization is induced within matrix vesicles (MVs). MVs are cell-derived extracellular membrane enclosed particles, about 0.1-1 micrometer in size (48). In a mineralization inducing environment, they are proposed to bud off the cell plasma membrane in a polarized fashion to the longitudinal septal matrix in the growth plate and to the newly formed osteoid under the mineral facing surfaces of osteoblasts in bone (48;54). MVs have been isolated from mineralizing odontoblasts, osteoblasts, chondrocytes and vascular smooth muscle cells (55-57). This is generally performed after a crude collagenase digestion (typically collagenase 500 U/ml at 37 °C for 3 hours). After gentle vortexing, MVs are then harvested by differential centrifugation. To this end, the collagenase digest is centrifuged at 13,000 x g for 20 minutes, and the resulting cells and cell debris are discarded. Subsequently, the supernatant is spun at 100,000 x g for 1 hour, which results in a pellet that contains MVs (58-62).

Many studies have been directed at the elucidation of the mechanism of MVs mineralization. In those studies, MVs isolated from tissues or cell culture systems were induced to calcify. This is generally performed by incubating isolated MVs in a so called synthetic cartilage lymph medium which is a physiological buffer containing approximately 2 mM calcium and 1.5 mM Pi (inorganic phosphate) (47;56;60;63-66). From these studies it was concluded that MVs have to be mineralization competent to nucleate calcium phosphate, because isolated MVs from non-mineralizing tissues do not calcify in synthetic cartilage lymph (59;67). Unlike the mineralization competent MVs, these latter MVs did not express Annexin V and had a lower APase activity (47;59). These observations suggest that not all MVs are equivalent and that only mineralizing tissue can produce mineralization competent MVs. These studies are complicated by the presence of vesicles derived from apoptotic cells (apoptotic bodies) in the matrix, which have properties similar but not identical to MVs (see next section) (61).

Several reports suggest that MVs do not contain crystals at the time of their release from the cell, but that the first crystalline mineral appears after the MV has been immobilized in selected areas of the collagen matrix (48). The mineral crystal is then formed by concentrating calcium and phosphate at the inner leaflet of the vesicle membrane, which has been reported to be enriched in phosphatidylserine (PS) (68). Once the mineral has reached a certain size it ruptures the vesicle membrane and contributes to the extracellular matrix (48). The mechanisms by which the crystals break down or penetrate through the membrane are not fully understood.

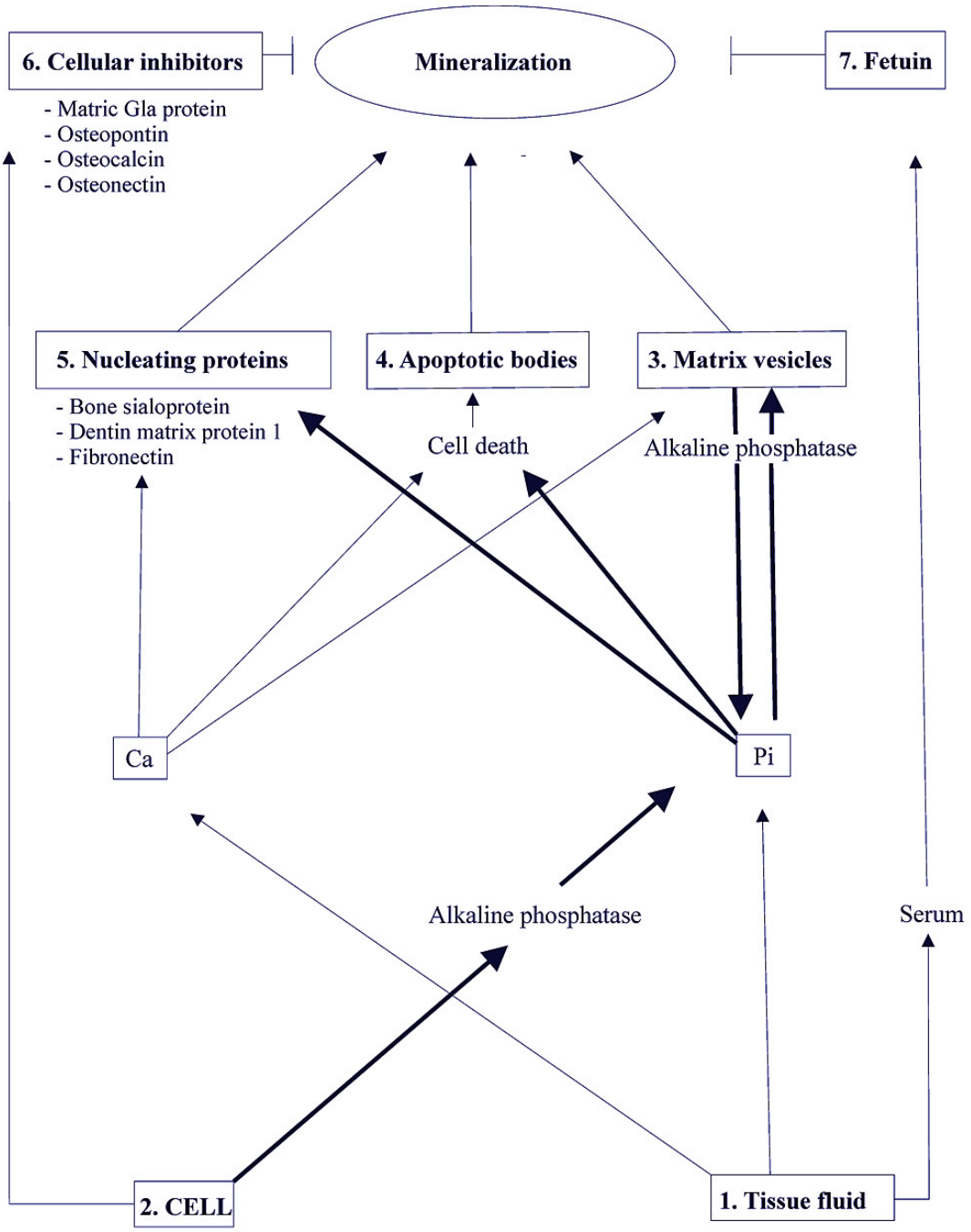

Figure 2. Schematic diagram of factors proposed to be involved in cell mediated mineralization. Bold arrows indicate that an increase of Pi rather than calcium induces cell-mediated mineralization. (1) Tissue fluid contains calcium and phosphate. An increase of Pi may induce mineralization. (2) Cells can also generate a local increase of Pi, when they increase APase-levels. The increased Pi induces: (3) the production of MVs, which contain APase and also generate Pi, (4) cell death, which results in the formation of apoptotic bodies, (5) production of nucleating proteins, which are secreted in the extracellular matrix. (6) To control mineralization, the cells constitutively produce mineralization inhibitors. (7) Tissue fluid also contains mineralization inhibitors, principally fetuin that originates from serum.

Because MVs are enclosed by a membrane, transport proteins are required to mediate the influx of mineral ions into these particles. Uptake of phosphate (Pi) is critical for the formation of minerals within MVs. It has been shown that the sodium dependent type III Pi transporter Glvr-1 is mainly expressed in the growth plate by early hypertrophic chondrocytes, which are the MVs producing cells (69). Furthermore, MVs isolated from chicken epiphyseal cartilage have been shown to contain a sodium dependent Pi transport system (70). However, other

studies report that MV mineralization is not strictly sodium dependent, suggesting the presence of other Pi transporters as well (63). MVs also have the potential to cleave phospho-compounds to inorganic phosphate, resulting in a local increase of Pi concentration, as APase has been shown to be enriched in the membrane of MVs (48;71).

Annexin II, V and VI have been reported to mediate the influx of calcium into the vesicles, by forming hexamers in the PS enriched MV membrane (72;73). Furthermore, Annexin V binds type II and X collagen and this interaction has been shown to stimulate its calcium channel activity (60). The role of Annexin V was further established through suppression by siRNA, which resulted in inhibited mineralization, while overexpression stimulated mineralization (74;75). Annexin expression has also been found to be increased in hypertrophic chondrocytes from the growth plate (76). However, Annexin V knockout mice did not show an impaired skeletal phenotype and neither were the *in vitro* calcification properties of the isolated annexin (-/-) chondrocytes significantly impaired (77). Possibly other members of the annexin family compensate the deficiency in annexin V.

4.2. The role of cell death

There is a strong correlation between mineralization and cell death. Especially pathological mineralization has often been associated with apoptotic or necrotic processes (11). Furthermore, terminally differentiated chondrocytes in the growth plate are deleted from the cartilage by programmed cell death (23;36). Inorganic phosphate (Pi), whose concentration is reported to increase from the proliferative to the hypertrophic zone in the growth plate, has been shown to be a potent apoptogen (78;79). A Pi-induced intracellular effect was evidenced, since Na-Pi transporter inhibitors were shown to inhibit apoptosis in parallel with mineralization. However, it should be noted that Pi-induced cell death was strongly synergized by the extracellular calcium concentration (69;80;81). A critical role of Pi in apoptosis was also established in mice affected with hypophosphatemia. These animals contain an expanded layer of late non hypertrophic chondrocytes in the growth plate, which is associated with a decrease in the number of apoptotic hypertrophic chondrocytes (82). In contrast, patients affected with hyperphosphatemia show pathological mineralization, which correlated with an increase in cell death (83;84).

Pi is not the only agent that induces cell death during mineralization, since pathological mineralization also occurs in the absence of hyperphosphatemia, indicating the role of additional factors. In agreement with this, it has been reported that no calcification occurred in vessels with calcium and Pi concentrations of 1.8 mM and 3.8 mM respectively, but mechanical injury resulted in extensive calcification under these conditions (85).

Until now it is not clear how apoptosis contributes to mineralization. Possibly, dying or injured cells may become highly permeable to calcium and phosphate, and may concentrate these ions beyond their solubility product, facilitating heterogeneous nucleation and crystal growth. It has been proposed that an early step in apoptosis is externalization of phosphatidylserine (PS) (86). PS has been shown to have a high affinity for calcium and may act as a nucleator for calcium phosphate crystal formation (36;87). However, it has been reported that mineral-PS interactions can retard crystal growth (88). This suggests that although PS has the capacity to nucleate calcium phosphate in cells undergoing apoptosis, other factors, probably produced by living cells, are necessary to induce crystal growth. It has also been suggested that apoptotic bodies derived from dying cells may act as nucleating mineralization centres in a similar way as described for MVs. Apoptotic bodies isolated from cell culture systems have been shown to precipitate calcium phosphate when incubated in a synthetic cartilage lymph (56;89-91). However, the precipitation capacity was less in apoptotic bodies when compared to MVs (56). This indicates that MVs have a stronger capacity to induce crystal growth, which was supported by Kirsch *et al.* (61) who reported that apoptotic bodies do not contain APase and the calcium channel forming annexins II, V and VI.

Inhibiting apoptosis with a general caspase inhibitor has been shown to inhibit mineralization in cell culture systems by approximately 40%, indicating a role of apoptosis in mineralization, but also a role of factors other than apoptosis in the process of mineralization (78;92).

Recently, it has been proposed that mineralizing hypertrophic growth plate chondrocytes are not dying by a classical form of apoptosis, because, in contrast to *in vitro* cell culture systems, they do not produce apoptotic bodies *in vivo* (23;93). Instead, it was speculated that they eliminate themselves by a process of autophagocytosis. This hypothesis is supported by ultramicroscopic examination of hypertrophic growth plate chondrocytes, showing that dying chondrocytes contain autophagic vacuoles (autophagosomes) and cell remnants that are blebbed off, indicative of autophagocytosis (23;93;94). This specific form of cell death is also called chondroptosis (93). Possibly, the blebs generated by these cells have mineralizing capacities.

4.3. The role of nucleating proteins

Mineralization has also been proposed to be regulated by non-collagenous proteins (NCPs) found in the organic matrix of bone (52;95-99). The NCPs are reported to constitute 5-10% of the total extracellular matrix and can be classified into four groups (Table 2). These groups include proteoglycans, glycoproteins, the γ carboxy glutamic acid (gla)-containing proteins and the serum associated proteins (95;99;100). Of these proteins mainly glycoproteins have been demonstrated to play a critical role in the initiation and growth of the calcium phosphate mineral phase (Table 3). Glycoproteins are proteins that are modified posttranslationally by glycosylation, phosphorylation and sulfatation. Some glycoproteins contain an RGD (Arg-Gly-Asp) sequence that interacts with the integrin receptor family. Glycoproteins that contain an RGD sequence are bone sialoprotein, BAG-75,

Table 2. The non-collagenous proteins regulating mineralization can be classified into four groups

Proteoglycans	Glycoproteins	γ carboxy glutamic acid (gla)-containing proteins	Serum proteins
versican decorin biglycan hyaluronate	osteonectin osteopontin bone sialoprotein BAG-75 dentin matrix protein-1 thrombospondin fibronectin vitronectin	osteocalcin matrix gla protein protein S	fetuin albumin growth factors

Table 3. Proteins that have been associated with mineralization

Protein	Mouse mutant	Phenotype	*In vitro* effect on mineralization	References
Annexin V	Anxa5 -/-	No obvious altered phenotype	Mediates influx of calcium into vesicles, siRNA inhibits mineralization	72-74, 77
Alkaline phosphatase	TNAP -/-	Hypophosphatemia and impaired growth	Release of inorganic Pi from organic phospho-compounds	71, 156, 157
Bone sialioprotein (BSP)	ND[1]	ND[1]	Nucleates calcium phosphate	51, 52, 158
BAG-75	ND[1]	ND[1]	Sequesters millimolar quantities of Pi	51, 52
Dentin matrix protein-1	dmp1 -/-	Decreased mineral to matrix ratio in bones	Facilitates hydroxyapatite crystal growth (nonphosphorylated)	95, 101, 111, 112
Fibronectin	Fn -/-	Non viable	Facilitates hydroxyapatite crystal growth	113, 114
Fetuin	Fetuin -/-	Vascular and soft tissue calcification	Inhibits precipitation of calcium and phosphate	119, 121, 122
Matrix GLA protein	MGP -/-	Vascular, valve and cartilage calcification	ND[1]	123
Osteopontin	OPN -/-	Enhanced valve implants calcification	inhibits mineralization when phosphorylated	128, 129
Osteonectin	ON -/-	Increased volume of adipose tissue	May retard crystal growth	96, 134, 135, 159
Osteocalcin	OC -/-	Increased bone mass	May retard crystal growth	96, 134-136

[1] ND: Not determined

dentin matrix protein-1, fibronectin, osteopontin and thromobospondin (99;101).

In vitro studies have shown that bone sialoprotein (BSP) can nucleate apatite crystals. BSP is an anionic phosphoprotein that is expressed almost exclusively in mineralized tissues (102;103). Furthermore, it has been demonstrated that after treatment with organophosphate for 4-8 hours, BSP localizes to the extracellular matrix in osteoblastic cultures, well before the first appearance of apatite crystals (104-106). This suggests that Pi (which is probably produced by APase) triggers BSP secretion into the extracellular matrix where it can subsequently nucleate calcium phosphate in metastable solutions. In addition, it has been reported that another noncollagenous bone matrix protein, bone acidic glycoprotein-75 (BAG-75) predicts the location of mineral nucleation, and possibly recruits BSP (51;52). Purified BAG-75 can self-associate into supramolecular spherical complexes and sequesters millimolar quantities of Pi, which indicates that BAG-75 generates a localized Pi source for crystal nucleation reactions (107). Interestingly, it has been proposed that BSP is associated with a population of vesicle-like structures (defined as crystal ghosts), which are 500-800 nm in size. However, BSP did not associate with the smaller 50-300 nm vesicle population (52). An important role of BSP in mineralization has been further established by the observation that transfection of BSP cDAN into non-mineralizing MC3T3-E1 subclones can restore the ability to form mineral deposits (51;108). On the basis of this information, BSP is likely to be involved with early mineral deposition. So far, the phenotype of BSP null mice is not known.

Another NCP, dentin matrix protein-1 (DMP1), has been reported to facilitate hydroxyapatite growth (95;100). A role of DMP1 in mineralization was suggested when it was shown to be mainly expressed during dentin mineralization, and later also in osteoblasts (109-111). Furthermore, DMP1 null mice have a decreased mineral to matrix ratio in bones (112). In an *in vitro* biomineralization model DMP1 has been shown to undergo a conformational change upon calcium binding and to subsequently assemble calcium phosphate nuclei into ordered protein-mineral complexes. This results in an inhibiting effect on spontaneous calcium phosphate precipitation. Thus, DMP1 could sequester and stabilize newly formed calcium phosphate clusters (95). In addition, another *in vitro* study reported that DMP-1-induced crystal growth and proliferation is dependent on its degree of phosphorylation, because nonphosphorylated DMP1 acts a nucleators while the phosphorylated form inhibits nucleation (101).

Another NCP, fibronectin also has been shown to facilitate hydroxyapatite growth in the presence of a hydroxyapatite seed, and a close association between fibronectin and hydroxyapatite has been found *in vivo* (113;114). Fibronectin, like DMP-1, has an inhibiting effect on spontaneous calcium phosphate precipitation (95;114). Therefore, it has been postulated that DMP-1 and fibronectin play a structural role in crystal growth, rather than a nucleating role.

4.4. The role of mineralization inhibitors

In mammals, mineralization is generally controlled by two serum NCPs, fetuin and matrix Gla protein (Table 3) (53;115). It has been proposed that the biological function of these proteins is to maintain high metastable blood calcium phosphate levels and to inhibit unwanted (soft tissue) mineralization. Fetuin is synthesized in the liver and found in high concentrations in mammalian serum. Because of its high affinity to hydroxyapatite it is

also found in bone and teeth (116-118). Fetuin knockout mice spontaneously develop widespread soft tissue calcifications, including significant myocardial calcification (119). In humans fetuin deficiency is associated with inflammation and vascular calcification (115;120). *In vitro* studies demonstrate that fetuin inhibits precipitation of supersaturated solution of calcium and Pi by formation of a high molecular mass fetuin-mineral complex (121). This complex prevents growth, aggregation and precipitation of calcium phosphate (122).

Matrix Gla protein is an extracellular matrix protein that is generally expressed by chondrocytes and vascular smooth muscle cells (123). Mice deficient in matrix Gla protein are normal at birth but develop severe calcification of all arteries (and cartilage) within weeks. These mice die at around 8 weeks of age, mostly due to a rupture of the aorta (123). Interestingly, it has recently been reported that MVs isolated from vascular smooth muscle cells that are induced to mineralize contain fetuin and matrix Gla protein (56;124). It has been speculated that this may be a defence mechanism of the cell to limit excessive mineralization (56;124).

The NCP osteopontin (OPN) has also been shown to control crystal growth (125). In addition, Pi has been proposed to be a specific signal for upregulation of OPN gene expression, which supports its regulatory role during mineralization (15;126). In agreement with this, gluteraldehyde fixed porcine aortic valves implanted into OPN null mice mineralized to a much greater extent than those implanted in wild type mice (127). However, the inhibiting effect of OPN on mineralization is dependent on the extent of phosphorylation of OPN, because OPN does not inhibit mineralization after dephosphorylation by APase (128-130). *In vivo,* OPN is a protein that is normally found in mineralized tissue, but also in epithelial lining cells of numerous organs, and body fluids, including urine, saliva, milk and bile (129;131). Next to inhibiting mineralization, OPN also regulates bone cell adhesion and osteoclast function in the skeleton (129). When OPN attaches to osteoclasts, it stimulates the acidification of the local environment, which will allow for the dissolution of the mineral (129).

Another NCP, osteonectin (ON), also known as SPARC (secreted protein, acidic, rich in cysteine), is a calcium binding matrix protein found in many tissues undergoing remodeling (132). Several *in vitro* studies have demonstrated that ON can inhibit crystal nucleation and retard crystal growth (133-135), although Hunter *et al.* (1996) found no effect (96). An explanation for the different results may be that different model systems were used, or the difference in ON concentrations tested. *In vivo,* ON deficient mice show an increased volume of adipose tissue and a decreased osteoblast and osteoclast number, resulting in osteopenia. This suggests that ON rather plays an important role in cell differentiation as well (132).

In vitro, similar results as with ON were obtained for the NCP osteocalcin (OC), which is also known as bone Gla protein (BGP) (96;99;133-135). In addition, *in vivo* transgenic OC deficient mice demonstrate an increase in bone mass, which suggests that OC indeed limits mineralization (136).

5. PERSPECTIVES

Pathological mineralization can have severe clinical consequences (Table 1). For example, articular cartilage calcification is one of the major degenerative diseases of the skeleton and leads to cartilage destruction, severe pain and joint stiffness (4). In addition, vascular calcification may lead to mortality (8;9). Although many investigations greatly contributed to a better understanding of the mechanisms regulating cell-mediated mineralization, many questions remain about the mechanisms that trigger cell-mediated mineralization and how this process is regulated. For instance, it is still not clear whether one type of vesicles induces mineralization, or whether more types of vesicles are involved. This might possibly be different between different tissues. In addition, it is unclear where the mineral exactly nucleates. For example, does the first mineral develop extracellularly in vesicles? Or does it start intracellularly, which will then result in the formation of vesicles? Furthermore, various proteins have been shown to be involved in mineralization, but it is unclear how these proteins are related mechanistically. Therefore, a detailed understanding of the roles of the various hypotheses and the coordinated interaction between them will provide novel therapeutic strategies to prevent pathological mineralization.

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