Published in final edited form as:

Calcif Tissue Int. 2006 November ; 79(5): 340–353.

Mechanism by which MLO-A5 Late Osteoblasts/Early Osteocytes Mineralize in Culture: Similarities with Mineralization of Lamellar Bone

C. Barragan-Adjemian¹, D. Nicolella², V. Dusevich¹, M. R. Dallas¹, J. D. Eick¹, and L. F. Bonewald¹

¹ Department of Oral Biology, University of Missouri at Kansas City, 650 East 25th Street, Kansas City, MO 64108-2784, USA

² Mechanical and Materials Engineering Division, Southwest Research Institute, 6220 Culebra Road, San Antonio, TX 78228-0510, USA

Abstract

The mechanisms whereby bone mineralizes are unclear. To study this process, we used a cell line, MLO-A5, which has highly elevated expression of markers of the late osteoblast such as alkaline phosphatase, bone sialoprotein, parathyroid hormone type 1 receptor, and osteocalcin and will mineralize in sheets, not nodules. In culture, markers of osteocytes and dendricity increase with time, features of differentiation from a late osteoblast to an early osteocyte. Mineral formation was examined using transmission electron microscopy, scanning electron microscopy with energy-dispersive X-ray analysis, and atomic force microscopy. At 3–4 days of culture, spheres of approximately 20–50 nm containing calcium and phosphorus were observed budding from and associated with developing cellular projections. By 5–6 days, these calcified spheres were associated with collagen fibrils, where over time they continued to enlarge and to engulf the collagen network. Coalescence of these mineralized spheres and collagen-mediated mineralization were responsible for the mineralization of the matrix. Similar calcified spheres were observed in cultured fetal rat calvarial cells and in murine lamellar bone. We propose that osteoid-osteocytes generate spherical structures that calcify during the budding process and are fully mineralized on their developing cellular processes. As the cellular process narrows in diameter, these mineralized structures become associated with and initiate collagen-mediated mineralization.

Keywords

MLO-A5 cells; Mineralization; Osteoblast; Osteoid; Osteocyte

Bone cells such as osteoblasts, osteoid-osteocytes, and osteocytes may play different roles in the initiation and regulation of bone mineralization. As early as 1976 and 1981, Bordier and coworkers [1] and Nijweide and coworkers [2] proposed that osteoid-osteocytes play an important role in the initiation and control of matrix calcification. Osteoid-osteocytes were described by Palumbo [3] in 1986 to be cells actively making matrix and calcifying this matrix. Like osteoblasts, their activity was polarized toward the mineralization front to which their cellular processes were oriented. Cellular processes oriented toward blood vessels only began to appear when mineralization began to spread around the cell. She described the cell body reducing in size in parallel with the formation of cytoplasmic processes. This reduction was about 30% at the osteoid-osteocyte stage and 70% with complete maturation of the osteocyte.

Owen [4] stated that during the time for an osteoblast to become an osteocyte, the cell has manufactured three times its own volume in matrix.

Mikuni-Takagaki and colleagues [5] proposed that casein kinase II, produced in high amounts by osteoid-osteocytes and not osteoblasts, is responsible for phosphorylation of the matrix proteins necessary for mineralization. Phosphoproteins appear to be essential for bone mineralization as evidenced by *in vitro* crystal nucleation assays [6,7] and by osteomalacia in animal models with deletion of specific genes such as dentin matrix protein 1 and *PHEX* (phosphate-regulating neutral endopeptidase on chromosome X) [8,9]. Deletion of inhibitors of mineralization such as sclerostin and osteoblast/osteocyte factor 45/MEPE (matrix extracellular phosphoglycoprotein) results in osteopetrosis [10,11]. Interestingly, these phosphoproteins that appear to regulate mineralization are expressed late in osteoblast differentiation and are all molecules that are highly expressed in osteocytes.

The two major events in mineralization are mineral crystal nucleation and mineral crystal propagation [12,13]. Several mechanisms have been proposed for the initiation and control of the nucleation event, such as matrix vesicles [14–17], whereas collagen is the accepted template for mineral crystal propagation. The pattern of mineral deposition associated with collagen is distinct. Crystals are deposited in a highly organized manner in the “hole” and overlap zones of collagen fibrils, and the newly formed crystals grow in length along their crystallographic c-axis parallel to the collagen fibril axes [18,19]. In addition, mineral deposition has been observed on the collagen fibril surface [20]. Collagen mineralization occurs after matrix vesicle deposition and can be related to or completely independent of it [21,22]. The factors that initiate nucleation of apatite on the collagen fibril are not clear; it has been proposed that matrix vesicles are responsible for providing the bond for the individual nucleation events and alignment of crystals on the collagen [19]. Lipids associated with both collagen and matrix vesicles may provide the link between vesicle and collagen-mediated mineralization [6]. This remains an area of active investigation.

MLO-A5 is a postosteoblast/preosteocyte-like cell line established from the long bones of 14-day-old mice expressing the large T antigen driven by the osteocalcin promoter [23]. These cells will mineralize in the absence of β -glycerolphosphate (β GP) in 6–7 days, but this process is accelerated by the addition of an external source of phosphate. Spectra obtained by Fourier transform infrared spectroscopy of these cultures are very similar to normal bone [23]. The cells do not express any markers of cartilage such as collagen type II, collagen type X, or aggrecan and therefore are not hypertrophic chondrocytes. They do express extremely high levels of alkaline phosphatase and osteocalcin as well as osteopontin, periostin, bone sialoprotein, and parathyroid hormone receptor compared to primary osteoblasts and osteoblast cell lines [23]. These cells were used for the present study in order to obtain insight into the potential mechanisms by which bone mineralizes.

Materials and Methods

Culture of Cells

MLO-A5 cells were cultured in 24-well plates at a density of $3.5 \times 10^4/\text{cm}^2$ in α -minimal essential medium (α -MEM) containing 5% fetal bovine serum (FBS) and 5% calf serum (CS). Upon confluence, designated day 0, media were removed and the cells were incubated in mineralizing media, α -MEM with 10% FBS, 5 mM β GP, 100 μ g/mL ascorbic acid. Media were changed every 2 days up to 12 days and cultures stopped at 3, 6, 9, and 12 days for analysis [23]. Fetal rat calvarial (FRC) cells were cultured as described previously [24].

von Kossa Staining for Phosphate and Quantitation

Cultured MLO-A5 cells were washed with phosphate-buffered saline (PBS), fixed with 10% buffered formalin for 10 minutes, and washed with water several times; a 2% silver nitrate solution was added, and then plates were exposed to ultraviolet light for 20 minutes, after which they were rinsed with water. Sodium thiosulfate (5%) was added for 3 minutes before rinsing with water. The modified van Gieson stain was then used as a counterstain after the von Kossa stain. Acid fuchsin solution (five parts of 1% acid fuchsin, 95 parts of picric acid) was added for 5 minutes before the plates were washed twice with 95% ethanol (ETOH) and twice with 100% ETOH, then air-dried for analysis. The mineralized areas and total area were measured using a semiautomated imaging system as described previously [25–27].

Alizarin Red Staining for Calcium and Quantitation

MLO-A5 cells were cultured for mineralization and fixed in formalin as mentioned above. Fixed cultures were washed three times with Tris-buffered saline (TBS) and then stained with 4 nM alizarin red S dye (AR-S) for 5 minutes. Cultures were then rinsed with water, followed by a 15-minute wash with TBS to reduce nonspecific AR-S stain [28]. The mineralized areas were measured using a semiautomated imaging system as described previously [25–27]. Bound AR-S dye was then extracted for 15 minutes with slow rotation at 4° C using an acid/alcohol solution (cold 10 mM HCl in 70% ETOH). The initial extract was collected and combined with a second extraction. The combined extracts were then diluted 1/5 in 100 mM phosphate buffer (pH 7.5), for reading at 520 nm. A standard curve for AR-S dye was performed for each analysis, and the amount of bound dye per culture well was determined.

Western Blot Analysis

MLO-A5 cells were cultured as described above, then washed with PBS. Radioimmunoprecipitation assay buffer (250 μ L) with proteinase inhibitors (phenylmethylsulfonyl fluoride 20 mM, leupeptin 20 nM, *N*-ethylmaleimide 100 mM) per well was added. The cell lysate was scraped, collected, and frozen at –80° C. The cell lysate was used to detect the expression of E11, a marker for early osteocytes, in the cultures. Protein content was determined using the Bio-Rad (Hercules, CA) protein assay. Separation was performed using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis mini-gels (Bio-Rad). Immunoblotting was performed as described previously [24,29]. Briefly, after transfer to the nitrocellulose, the background was blocked with 5% bovine serum albumin + 1% nonfat milk + 0.05% NaN₃ in PBS for 2 hours at room temperature; washed with PBS; incubated in anti-8.1.1 and primary antibody 1:2,500 in 1% milk, 0.05% NaN₃ overnight at 4° C; washed with PBS and phosphate buffered saline Tween-20 (PBST); and incubated in secondary antibody, per-oxidase-conjugated goat anti-hamster immunoglobulin G (IgG 1:5,000) in 5% milk-PBS, NaN₃-free. The immunostained bands were visualized using an enhanced chemiluminescence detection system following the manufacturer's instructions (Super Signal West Dura; Pierce, Rockford, IL). The E11 antibody, 8.1.1, was a kind gift of Dr. Andrew Farr, University of Washington, Seattle, WA, USA [30,31].

Immunohistochemical Staining for Collagen Type I

MLO-A5 cells were plated in Lab-Tek (Naperville, IL) chamber slides at a density of $2 \times 10^4/\text{cm}^2$ and cultured as described above. At the end of culture, cells were washed with PBS twice, fixed with 95% ETOH for 5 minutes, and washed with PBS three times. After the washes, cultures were incubated with blocking solution (PBS + 1% horse serum + 0.05% NaN₃) for 2 hours at room temperature, followed by incubation with polyclonal antibody to type I collagen, LF-67, which recognizes the C-telopeptide of collagen type I (antibody kindly provided by Dr. Larry W. Fisher, National Institutes of Health, Bethesda, MD). A 1:400 dilution in PBS + horse serum was used for 1 hour at room temperature, followed by incubation with Cy-3 conjugated

donkey anti-rabbit IgG in blocking solution 1:250 for 1 hour and washing with PBS six times. The cells were mounted with 9:1 glycerol:PBS + 5% *N*-propylgallate to reduce photobleaching and then examined under fluorescence using a Nikon (Tokyo, Japan) eclipse E800 microscope [24].

Transmission Electron Microscopy

MLO-A5 cells were cultured on Corning (Corning, NY) six-well plates at a density of $2 \times 10^4/\text{cm}^2$. At the end of culture, cells were washed three times with PBS and fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer [32] for 1 hour at room temperature. Samples were washed three times with 0.1 M sodium cacodylate buffer and postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 hour at room temperature. Samples were then dehydrated with ETOH series and processed for embedding in epon resin (Poly/bed 812; Polysciences, Warrington, PA) for 2 hours. Complete polymerization was performed in a 60°C oven for 24 hours. Thin sections were then cut using a diamond knife and mounted on copper grids (300 mesh). Sections were stained with uranyl acetate and lead citrate and examined using a Philips (Hamburg, Germany) CM12 STEM [33].

Preparation of Murine Bone for Transmission Electron Microscopy

Three-month-old wild-type C57BL/6 mice were killed using a protocol approved by the Institutional Animal Care and Use Committee of the University of Missouri-Kansas City. Ulnas were dissected and fixed in 2% paraformaldehyde and 2% glutaraldehyde solution buffered at pH 7.4 with 0.1 M sodium cacodylate for 8 hours. Samples were washed three times with 0.1 M sodium cacodylate buffer and postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 hour at room temperature. The rest of the embedding procedure was performed as described above. After sections were cut, the remaining block was imaged using scanning electron microscopy (Nalge Nunc Int., Rochester, NY) (SEM).

SEM

MLO-A5 cells were cultured on Thermanox coverslips (Nalge Nunc Int., Rochester, NY), $3.5 \times 10^4/\text{cm}^2$ in 24-well plates, as described. At the end of the culture, cells were gently washed with PBS and fixed with 10% formalin for 20 minutes, washed again with PBS, dehydrated in a graded series of ETOH, and dried using hexamethyl disilazone for 5 minutes. After dehydration, the coverslips were attached to a stub and sputter-coated with gold palladium. The gold palladium-coated cultures were examined using an FEI/Philips XL30 field emission environmental SEM. Accelerated voltage in the range 15–25 KeV was used for the secondary and backscatter electron imaging. For X-ray microanalysis (energy-dispersive spectrometry [EDS]), cultures were carbon-coated and examined with 15 KeV accelerating voltage. X-ray spectra and maps for calcium and phosphorus distribution were acquired [33,34].

Atomic Force Microscopy

MLO-A5 cells were cultured on Thermanox coverslips, $3.5 \times 10^4/\text{cm}^2$ in 24-well plates, and treated for mineralization as described above. At the end of culture, cells were rinsed with PBS, fixed with 10% formalin for 20 minutes, and washed with PBS. Coverslips were carefully removed from the culture wells and placed on microscope slides for atomic force microscopy (AFM) examination. Cultures were examined using a Digital Instruments Dimension 3000 Atomic Force Microscope (Veeco Digital Instruments, Woodbury, NY) operating in noncontact (tapping) mode, which allowed simultaneous capture of a height image, which describes the topology of the culture, and a phase image, which describes the material characteristics of the culture. Images were acquired using either a $10 \times 10 \mu\text{m}$ or a $3 \times 3 \mu\text{m}$ scan size.

Results

von Kossa staining shows a “fractal” or “honeycomb” staining pattern for MLO-A5 cells, not nodular as for primary osteoblasts and other osteoblast cell lines (Fig. 1). This process is accelerated 3–5 days in the presence of β GP; therefore, data are shown using these culture conditions. Similar patterns of mineralization were observed without β GP but delayed [23]. Figure 1 shows a time course for mineral formation of 3–12 days. At 3 days, the initial collagen matrix begins to form. At 4–6 days, the extracellular matrix (ECM) begins to mineralize. At this stage, honeycomb-like structures are present and collagen type I is observed with van Gieson counterstain. At 9 days, the honeycomb-like matrix is highly mineralized, covering the entire well in a fractal pattern that is intensified by 12 days. The figure shows the nodular staining of primary FRC osteoblasts cultured for 2 weeks [24] and BMP-2-treated 2T3 osteoblast-like cells cultured for 2 weeks [35].

Quantification of mineralization as shown by von Kossa stain in Figure 1 was validated using AR-S staining. von Kossa detects phosphate, whereas AR-S detects calcium [32]. As can be seen in Figure 2, staining increased with extended time in culture.

Analysis of the ultrastructure of the cultures using SEM was performed to examine the initiation, structure, distribution, and composition of the mineralized matrix. At 9 days, secondary electron images show a dense ECM composed of mineralized and nonmineralized matrix (Fig. 3A). Cells can be seen on top of the matrix, and their processes follow the honeycomb pattern (Fig. 3B). A mineralized honeycomb-like matrix is present (Fig. 3C) shown by backscatter electron microscopy that highlights mineral of high density compared with the nonmineralized matrix. Since the main mineral component of bone is hydroxyapatite ($\text{Ca}_{10}[\text{PO}_4]_6[\text{OH}]_2$) with trace amounts of carbonate, magnesium, sodium, fluoride, citrate, iron, zinc, silicon, and pyrophosphate, the formation and accumulation of calcium and phosphate during the initiation of mineralization is critical [19]. Therefore, calcium and phosphorus maps were obtained from the cultures at 9 days to verify the presence of bone-like mineral (Fig. 4). Calcium (Fig. 4A) and phosphorus (Fig. 4C) colocalize in the mineralized structure (Fig. 4B). The presence of mineral was confirmed in the selected area by EDS exhibiting a Ca/P peak ratio of approximately 1.6 (Fig. 4D). This value is in agreement with that found in physiologically calcified tendons [34]. The calcium spectra were also analyzed in MLO-A5 cultures and found to be positive (data not shown) [13].

To determine if MLO-A5 cells differentiate in culture into osteocyte-like cells, a Western blot analysis for the osteocyte-specific antigen E11 [36–38] was performed. At 0 days, no band was detectable in MLO-A5 cultures, but a faint band was detectable at 3 days. Over time in culture, MLO-A5 cells began to express higher amounts of E11, with highest expression at days 6 and 9 (Fig. 5). MLO-Y4 osteocyte-like cells [27] were used as a positive control.

Collagen type I is essential for normal mineralization of bone [19]. Only collagen type I, and not type II or X, was found in these cultures, as shown previously [23]. Immunofluorescent staining for type I collagen was performed on MLO-A5 cells from 0 to 12 days of culture (Fig. 6). The antibody LF-67 is specific for the telopeptide of type I collagen, which though cleaved will be retained in the matrix. At day 0, staining appears only intracellularly in the cytoplasm. At 3 days after addition of ascorbic acid and β GP, collagen type I is secreted into the ECM following a fibrillar pattern to initiate the beginnings of a collagen network. During this time, cell proliferation is increased and collagen secretion continues. The pattern is clearly fibrillar at 6 days, similar to the von Kossa stain (Fig. 1) and the backscatter image (Fig. 3C), but becomes more amorphous at 9 and 12 days, when the ECM is highly mineralized, suggesting that the antibody can only penetrate the top layer of the cultures and may only recognize collagen present in cells on the top layer of the mineralized surface. Collagen production was

also examined by transmission electron microscopy (TEM), SEM, and AFM (Fig. 7). By TEM, collagen type I fibers were observed between cell layers (Fig. 7A). The collagen shows the characteristic 64–70 nm periodicity as shown by both AFM (Fig. 7B) and SEM (Fig. 7C). Mineralized collagen fibers (Fig. 7D) were observed with time. Mineralized collagen fibers were also observed by TEM (Fig. 8). Collagen type I regulates the extent to which mineral can propagate [22]. As shown in the electron photomicrograph in Figure 8A, mineral is formed on top of and along the collagen network and is only present in areas of matrix containing collagen. Mineral is formed not only on the surface of the collagen but also within and between the fibers (Fig. 8B). Small, mineralized, spherical structures were also observed in MLO-A5 cultures by TEM (Fig. 8C). These structures, which resemble calcospherites of 100–250 nm in size, were found close to cell membranes.

These calcified spheres were also observed by SEM (Fig. 9) and followed over time in culture. When cells reach confluence at day 0, β GP and ascorbic acid were added to the media to accelerate matrix and mineral formation. At this early stage, backscatter image analysis did not show any evidence of mineralization. However, some nonmineralized protrusions or blebs on the cell membrane were observed, suggesting that spherical structures are initially unmineralized and mineralize later (Fig. 9, 0 days). At 3–6 days, mineralized spherical structures are observed associated with cellular processes. Backscatter imaging showed that these spherical structures have a higher density than the surrounding cell or matrix. EDS was performed on three different spherical structures and on the less dense cytoplasm in triplicate to determine if both calcium and phosphorus are present and colocalize in these spherical structures. The top spectra represent the background and the bottom spectra are overlaid analysis of six different spherical structures. Clearly, calcium and phosphorus were present in the spherical structures at a peak ratio of 1.6 and are absent from other areas of the matrix. At 6 days, these structures could be observed budding from the cell membrane (Fig. 9, 6 days). Note that the upper bud (arrow) appears to be calcifying while budding. Potential budding processes were also observed at 0 days. These observations suggest that spherical structures mineralize while budding and while still associated with the cell membrane and are the initiators of mineralization in this cell model. At 7–9 days, the spherical structures were associated with the collagen network (Fig. 9, 9 days). The spherical structures increased in size (200–250 nm), coalesced, and engulfed collagen fibrils, as shown at day 9.

Secondary electron imaging at 6 days of culture showed that the spherical structures were budding (arrow) from the cell process (Fig. 10A) and mineralized, as shown by backscatter micrographs (Fig. 10B). Some spherical structures were not visible by SEM in Figure 10A yet became visible beneath the cell process by EDS (Fig. 10B). The spherical structures appeared to expand and engulf collagen fibers, as shown in Figure 10C, where a single sphere appears to be engulfing at least four or five collagen fibers. Figure 10D shows the spheres at various stages of growth in size and in association with the collagen network. The spheres appear to coalesce into much larger mineralized structures. AFM shows a similar pattern of spherical structures containing plate-like crystals interspersed between collagen fibers (Fig. 11). Experiments were performed previously showing that the structures are not nanobacteria or macromolecular mineralization [39].

Similar calcified spheres to the ones formed in MLO-A5 cells were observed by SEM in FRC cells cultured for 20 days with the addition of BMP-2, β GP, and ascorbic acid. Figure 12 shows FRC culture where collagen fibrils and the calcified spheres were always colocalized.

Figure 13A is a TEM image of an osteoid-osteocyte partially surrounded by mineral in lamellar bone in mice. Small mineralized structures of 50–100 nm (Fig. 13B, arrowhead) were observed in the osteoid frequently associated with the dendritic processes of these cells (Fig. 13B, arrow). A size gradient can be seen where the initial smallest mineralized structures are scattered

throughout the osteoid and appear to increase in size approaching and through the mineralization front. These structures appear to coalesce and eventually surround the embedding cell. Different stages of the embedding cell can be visualized by SEM, as shown in Figure 14. Figure 14A shows a cell beginning to embed in osteoid. Small mineralized spheres are seen toward the mineralization front, 100–200 nm, that increase in size, 500 nm and larger. Figure 14B and C shows osteoid-osteocytes partially surrounded by this size gradient of mineralized spheres, while Figure 14D shows the fully embedded cell completely surrounded by mineral. The distinct mineralized structures can no longer be delineated from each other at this stage.

Figure 15 shows a diagram of our proposed mechanism whereby an osteoid-osteocyte regulates the mineralization process. The embedding cell simultaneously produces collagen and fully mineralized structures of 20–50 nm that become associated with collagen as the cellular processes become narrower in diameter. These mineralized structures continue to grow in size from 50 nm to 1 μ m or larger, thereby engulfing the collagen fibers and coalescing to form the mineralized matrix.

Discussion

Previously, we described and characterized the MLO-A5 cell line and its capacity to rapidly generate a bone-like matrix compared to other cell lines and to primary fetal calvarial cells [23]. Here, we show that MLO-A5 cells mineralize in sheets in culture, not in nodules, and that this mineralized matrix contains a ratio of calcium to phosphorus similar to bone. We have also identified small mineralized spheres of 50–200 nm that appear early in culture at 3–6 days, associated with the cell membrane, also with a calcium to phosphate ratio similar to bone. Similar structures were observed in mouse lamellar bone.

Several mechanisms are thought to be responsible for mineralization of bone ECM. Initiation of crystal formation occurs at multiple sites in the ECM, followed by oriented crystal growth, crystal proliferation, and crystal maturation [20]. It is thought that matrix vesicles and/or collagen in conjunction with anionic matrix proteins initiate and control this process. In MLO-A5 cells, large amounts of collagen are simultaneously produced as the mineralized spheres dissociate from the cell membrane and associate with the newly generated collagen fibers. Once associated with collagen, they appear to grow and engulf the collagen fibers, reaching sizes as large as 500 nm before coalescing to form the mineralized matrix. We also examined bone formation *in vivo* for the presence of these mineralized spheres. Forming lamellar bone, where osteoblasts differentiate into osteoid-osteocytes, revealed similar structures of similar sizes. The first small structures of 20–200 nm appear in the osteoid, which increase in size and coalesce to form the mineralized bone. These observations led us to propose that lamellar bone may mineralize via this process.

The MLO-A5 cell line is homogeneous compared to primary cells, which most likely explains the homogeneous sheets of mineral formed in culture compared to nodules that form in primary cell culture. Also, these cells appear poised to rapidly secrete collagen and rapidly mineralize their matrix. The cells express extremely high levels of alkaline phosphatase and bone sialoprotein compared to other cell lines and primary osteoblasts, therefore representing a late osteoblast stage of differentiation [23]. Here, we show that these cells begin to express markers of osteocytes such as E11, a membrane molecule highly expressed in the embedding osteocyte [36,38] which increases with time and mineralization in culture. We found that this molecule plays a role in dendrite elongation in response to fluid flow shear stress [38]. MLO-A5 cells begin to express the characteristics of osteoid-osteocytes, and it is at this stage that the small mineralized spheres appear associated with newly generated cellular projections suggestive of dendritic processes. The osteoblast to osteocyte transformation has been relatively unexplored.

The function of osteoid-osteocytes has recently been reviewed by Franz-Odenaal and colleagues [40]. These authors suggest that this transformation should not be viewed as the end of a process but as a continuum of differentiation. MLO-A5 cells should prove a useful model to study not only the process of mineralization but also the transformation process of the osteoblast into an osteocyte.

Three processes have been described for the initiation and control of mineralization: (1) matrix vesicles, (2) “crystal ghosts,” and (3) bone mineralization foci. Clearly, matrix vesicles play a crucial role in the mineralization of cartilage, as described over 30 years ago by Anderson [41] and Bonucci [42] as either “vesicles” or “calcifying globules” in epiphyseal cartilage. They were described as membrane-invested, extracellular spheres of about 200 nm formed by budding from the cell surface membrane. It was originally thought that these vesicles, devoid of mineral, are deposited or “left-behind” in the newly forming matrix to act as nucleators of mineralization in a front distant from the chondrocyte or osteoblast [43]. The lipid composition of matrix vesicles is clearly different from the cell membrane, and alkaline phosphatase and other factors are highly elevated in this organelle [44]. These spherical membrane vesicles appear to be sites of first mineralization and to contain factors that are responsible for initiating apatite crystal formation [14]. The crystals inside the vesicles are randomly deposited in radial-shaped spherular clusters [22], which then appear to rupture the membrane and form calcospherites associated with collagen [44].

“Crystal ghosts” were described by Bonnucci as organic structures masked by mineral that only become recognizable after decalcification [17]. In cartilage, these structures are related to acid proteoglycans. Organic structures have been reported in round aggregates of crystals called “mineralization nodules” (not to be confused with “mineralizing nodules” [45] in cultures of primary osteoblasts that are visible to the naked eye). These structures have also been called “calcifying nodules,” range in size from 0.1 to 0.3 μm and up to 3 μm in diameter in bone in remodeling haversian canals [46], and have been described as “calcified microspheres” in bovine bone [47]. It may be that the structures described in the present study are related to these structures. How crystal ghosts are formed is still the subject of investigation.

Midura et al. [28] described large structures of 10–20 μm , the size of a cell, called “bone mineralization foci” (BMF) that appear to play a role in initiating mineralization in intramembranous osteogenesis. They described these structures in woven bone, in the marrow ablation model, and in UMR106 cells [48]. They proposed that BMF act to sequester proteins within a protective environment for the purpose of mineral nucleation (potentially in matrix vesicles located within these structures). Once nucleation has occurred, the BMF structure is ruptured and mineral propagation along collagen fibrils can occur. Therefore, the biomineralization process may be initiated and propagated by different mechanisms in different types of bone. Others [13] have observed a continuum of mineralization foci within newly forming bone induced by marrow ablation, ranging from matrix vesicles to calcospherites like those observed in the present study. The processes responsible for biomineralization of woven compared to lamellar bone, endochondral compared to intramembranous bone, and embryonic compared to adult bone formation are expected to share some but also to have distinct and different molecular pathways and underlying mechanisms. Here, we propose a third mechanism for mineralization, appropriate for lamellar bone.

By TEM, we could only identify already mineralized spheres on cell membranes, not typical matrix vesicles. These mineralized spheres resemble the “mineralized matrix” in cultures of primary osteoblasts as described by Nanci and coworkers [33, see their Fig. 9C]. By SEM, the small mineralized spheres in the present study very closely resemble the “small globular masses” in 21-day cultures of primary bone cells (see Fig. 7 of Nanci et al. [33] and our present Fig. 11). Nanci et al. stated that regions containing these masses show an accumulation of

calcium and phosphate and refer to similar structures as “patches” *in vivo* [33,49]. We pinpointed calcium and phosphate to similar structures in MLO-A5 cells at 4–6 days of culture.

It has been difficult to characterize the mineralization process. Use of cell lines and new technologies such as SEM and AFM are allowing investigation at the nanoscale. TEM has been the major technique used to visualize and study matrix vesicles, not SEM [14]. Whereas with TEM incipient mineral is lost and only a two-dimensional image is obtained, with SEM and AFM labile mineral is retained and these techniques provide a three-dimensional image. With TEM the sample is sectioned and only a narrow plane can be examined; therefore, structures can be easily missed. Using SEM, TEM, and AFM in our study, the following were visualized: (1) collagen fibers and networks, (2) small mineralized spheres associated with either cell processes or collagen networks, (3) growth and enlargement of these mineralized spheres, and (4) their engulfment of collagen fibers.

With cell lines, one must always be conscious of artifact. We performed experiments to verify that the small spherical structures were not macromolecules such as nanoparticles [50] or an artifact of culturing with serum [51]. These macromolecules were not visible until 30 days of cell-free culture and are much smaller than the mineralized spheres observed at 4–6 days of culture of MLO-A5 cells [39]. Therefore, the structures in MLO-A5 cultures are not nanobacteria or serum macromolecules. More importantly, others have observed similar structures produced by primary bone cells. TEM and SEM images of primary cultures of osteoblasts, as shown by Nanci et al. [33], are very similar to images of MLO-A5 cultures, as shown in the present study; and both studies found these structures to contain calcium and phosphate by EDS.

During her three-dimensional study of osteoid-osteocytes, Palumbo [3] observed matrix vesicles left by osteoblasts but could not find these structures associated with the cellular processes of osteoid-osteocytes. However, she still ascribed regulation of the mineralization process to osteoid-osteocytes, especially on the mineral-facing side, possibly in conjunction with matrix vesicles left behind by osteoblasts. She proposed that the cellular processes on the mineral-facing side are involved in bone formation and those on the vascular side, in cell nutrition. Our studies support the hypothesis that the osteoid-osteocyte is responsible for mineralization, as proposed by others [1–3]; and we propose a mechanism for this process in lamellar bone. This mechanism includes the simultaneous generation of cellular processes and mineralized spheres associated with the cell membrane that release from the membrane and associate with, expand, and engulf collagen fibrils.

Acknowledgements

We acknowledge the help of Dr. Jian Feng in the preparation of the murine bone. This study was supported by National Institutes of Health grants AR46798 and DE07294.

References

1. Bordier, MP.; Miravet, L.; Ryckerwaert, A.; Rasmussen, H. Morphological and morphometrical characteristics of the mineralization front. A vitamin D regulated sequence of bone remodeling. In: Meunier, PJ., editor. Bone Histomorphometry. Armour Montagu; Paris: 1976. p. 335-354.
2. Nijweide PJ, van der Plas A, Scherft JP. Biochemical and histological studies on various bone cell preparations. *Calcif Tissue Int* 1981;33:529–540. [PubMed: 6274487]
3. Palumbo C. A three-dimensional ultrastructural study of osteoid-osteocytes in the tibia of chick embryos. *Cell Tissue Res* 1986;246:125–131. [PubMed: 3779795]
4. Owen M. Cell population kinetics of an osteogenic tissue I. *Clin Orthop Relat Res* 1995;313:3–7. [PubMed: 7641495]

5. Mikuni-Takagaki Y, Kakai Y, Satoyoshi M, Kawano E, Suzuki Y, Kawase T, Saito S. Matrix mineralization and the differentiation of osteocyte-like cells in culture. *J Bone Miner Res* 1995;10:231–242. [PubMed: 7754802]
6. Boskey AL. Matrix proteins and mineralization: an overview. *Connect Tissue Res* 1996;35:357–363. [PubMed: 9084675]
7. Hunter GK, Hauschka PV, Poole AR, Rosenberg LC, Goldberg HA. Nucleation and inhibition of hydroxyapatite formation by mineralized tissue proteins. *Biochem J* 1996;317(pt 1):59–64. [PubMed: 8694787]
8. Blair HJ, Gormally E, Uwechue IC, Boyd Y. Mouse mutants carrying deletions that remove the genes mutated in Coffin-Lowry syndrome and lactic acidosis. *Hum Mol Genet* 1998;7:549–555. [PubMed: 9467016]
9. Strom TM, Francis F, Lorenz B, Boddreich A, Econs MJ, Lehrach H, Meitinger T. *Pex* gene deletions in Gy and Hyp mice provide mouse models for X-linked hypo-phosphatemia. *Hum Mol Genet* 1997;6:165–171. [PubMed: 9063736]
10. Gowen LC, Petersen DN, Mansolf AL, Qi H, Stock JL, Tkalecic GT, Simmons HA, Crawford DT, Chidsey-Frink KL, Ke HZ, McNeish JD, Brown TA. Targeted disruption of the osteoblast/osteocyte factor 45 gene (*OF45*) results in increased bone formation and bone mass. *J Biol Chem* 2003;278:1998–2007. [PubMed: 12421822]
11. Winkler DG, Sutherland MK, Geoghegan JC, Yu C, Hayes T, Skonier JE, Shpektor D, Jonas M, Kovacevich BR, Staehling-Hampton K, Appleby M, Brunkow ME, Latham JA. Osteocyte control of bone formation via sclerostin, a novel BMP antagonist. *EMBO J* 2003;22:6267–6276. [PubMed: 14633986]
12. Boskey AL. Biomineralization: conflicts, challenges, and opportunities. *J Cell Biochem Suppl* 1998;30–31:83–91.
13. Sela, J.; Schwartz, Z.; Swain, LD.; Boyan, BD. *The Role of Matrix Vesicles in Calcification*. CRC Press; Boca Raton, FL: 1992.
14. Anderson HC. Vesicles associated with calcification in the matrix of epiphyseal cartilage. *J Cell Biol* 1969;41:59–72. [PubMed: 5775794]
15. Anderson HC. Molecular biology of matrix vesicles. *Clin Orthop Relat Res* 1995;314:266–280. [PubMed: 7634645]
16. Bonucci E. Presence of “crystal ghosts” in bone nodules. *Calcif Tissue Int* 1979;29:181–182. [PubMed: 116760]
17. Bonucci E. Crystal ghosts and biological mineralization: fancy spectres in an old castle, or neglected structures worthy of belief? *J Bone Miner Metab* 2002;20:249–265. [PubMed: 12203030]
18. Glimcher MJ. Mechanism of calcification: role of collagen fibrils and collagen-phosphoprotein complexes in vitro and in vivo. *Anat Rec* 1989;224:139–153. [PubMed: 2672881]
19. Landis WJ. An overview of vertebrate mineralization with emphasis on collagen-mineral interaction. *Gravit Space Biol Bull* 1999;12:15–26. [PubMed: 11541779]
20. Landis WJ, Hodgins KJ, Song MJ, Arena J, Kiyonaga S, Marko M, Owen C, McEwen BF. Mineralization of collagen may occur on fibril surfaces: evidence from conventional and high-voltage electron microscopy and three-dimensional imaging. *J Struct Biol* 1996;117:24–35. [PubMed: 8776885]
21. Arsenault AL, Kohler DM. Image analysis of the extracellular matrix. *Microsc Res Tech* 1994;28:409–421. [PubMed: 7919528]
22. Landis WJ, Silver FH. The structure and function of normally mineralizing avian tendons. *Comp Biochem Physiol A Mol Integr Physiol* 2002;133:1135–1157. [PubMed: 12485697]
23. Kato Y, Boskey A, Spevak L, Dallas M, Hori M, Bonewald LF. Establishment of an osteoid preosteocyte-like cell MLO-A5 that spontaneously mineralizes in culture. *J Bone Miner Res* 2001;16:1622–1633. [PubMed: 11547831]
24. Dallas SL, Keene DR, Bruder SP, Saharinen J, Sakai LY, Mundy GR, Bonewald LF. Role of the latent transforming growth factor beta binding protein 1 in fibrillin-containing microfibrils in bone cells in vitro and in vivo. *J Bone Miner Res* 2000;15:68–81. [PubMed: 10646116]
25. Dean DD, Schwartz Z, Bonewald L, Muniz OE, Morales S, Gomez R, Brooks BP, Qiao M, Howell DS, Boyan BD. Matrix vesicles produced by osteoblast-like cells in culture become significantly

- enriched in proteoglycan-degrading metalloproteinases after addition of beta-glycerophosphate and ascorbic acid. *Calcif Tissue Int* 1994;54:399–408. [PubMed: 8062158]
26. Chen D, Ji X, Harris MA, Feng JQ, Karsenty G, Celeste AJ, Rosen V, Mundy GR, Harris SE. Differential roles for bone morphogenetic protein (BMP) receptor type IB and IA in differentiation and specification of mesenchymal precursor cells to osteoblast and adipocyte lineages. *J Cell Biol* 1998;142:295–305. [PubMed: 9660882]
 27. Kato Y, Windle JJ, Koop BA, Mundy GR, Bonewald LF. Establishment of an osteocyte-like cell line, MLO-Y4. *J Bone Miner Res* 1997;12:2014–2023. [PubMed: 9421234]
 28. Midura RJ, Wang A, Lovitch D, Law D, Powell K, Gorski JP. Bone acidic glycoprotein-75 delineates the extracellular sites of future bone sialoprotein accumulation and apatite nucleation in osteoblastic cultures. *J Biol Chem* 2004;279:25464–25473. [PubMed: 15004030]
 29. Bonewald LF, Wakefield L, Oreffo RO, Escobedo A, Twardzik DR, Mundy GR. Latent forms of transforming growth factor-beta (TGF- β) derived from bone cultures: identification of a naturally occurring 100-kDa complex with similarity to recombinant latent TGF- β . *Mol Endocrinol* 1991;5:741–751. [PubMed: 1922093]
 30. Farr A, Nelson A, Hosier S. Characterization of an antigenic determinant preferentially expressed by type I epithelial cells in the murine thymus. *J Histochem Cytochem* 1992;40:651–664. [PubMed: 1374092]
 31. Farr AG, Berry ML, Kim A, Nelson AJ, Welch MP, Aruffo A. Characterization and cloning of a novel glycoprotein expressed by stromal cells in T-dependent areas of peripheral lymphoid tissues. *J Exp Med* 1992;176:1477–1482. [PubMed: 1402691]
 32. Bonewald LF, Harris SE, Rosser J, Dallas MR, Dallas SL, Camacho NP, Boyan B, Boskey A. von Kossa staining alone is not sufficient to confirm that mineralization in vitro represents bone formation. *Calcif Tissue Int* 2003;72:537–547. [PubMed: 12724828]
 33. Nanci A, Zalzal S, Gotoh Y, McKee MD. Ultra-structural characterization and immunolocalization of osteopontin in rat calvarial osteoblast primary cultures. *Microsc Res Tech* 1996;33:214–231. [PubMed: 8845520]
 34. Arsenault AL. Crystal-collagen relationships in calcified turkey leg tendons visualized by selected-area dark field electron microscopy. *Calcif Tissue Int* 1988;43:202–212. [PubMed: 3145125]
 35. Ghosh-Choudhury N, Windle JJ, Koop BA, Harris MA, Guerrero DL, Wozney JM, Mundy GR, Harris SE. Immortalized murine osteoblasts derived from BMP 2-T-antigen expressing transgenic mice. *Endocrinology* 1996;137:331–339. [PubMed: 8536632]
 36. Schulze E, Witt M, Kasper M, Lowik CW, Funk RH. Immunohistochemical investigations on the differentiation marker protein E11 in rat calvaria, calvaria cell culture and the osteoblastic cell line ROS 17/2.8. *Histochem Cell Biol* 1999;111:61–69. [PubMed: 9930885]
 37. Wetterwald A, Hoffstetter W, Cecchini MG, Lanske B, Wagner C, Fleisch H, Atkinson M. Characterization and cloning of the E11 antigen, a marker expressed by rat osteoblasts and osteocytes. *Bone* 1996;18:125–132. [PubMed: 8833206]
 38. Zhang K, Barragan-Adjemian C, Ye L, Kotha S, Dallas M, Lu Y, Zhao S, Harris M, Harris SE, Feng JQ, Bonewald LF. E11/gp38 selective expression in osteocytes: regulation by mechanical strain and role in dendrite elongation. *Mol Cell Biol* 2006;26:4539–4552. [PubMed: 16738320]
 39. Barragan-Adjemian, C.; Nicolella, D.; Dusevich, V.; Dallas, M.; Eick, JD.; Bonewald, L. A proposed model for the mineralization of bone. In: Landis, WJ.; Sodek, J., editors. 8th International Conference on the Chemistry and Biology of Mineralized Tissues; Banff, Alberta, Canada. 2004. p. 47-50.
 40. Franz-Odenaal TA, Hall BK, Witten PE. Buried alive: how osteoblasts become osteocytes. *Dev Dyn* 2006;235:176–190. [PubMed: 16258960]
 41. Anderson HC. Electron microscopic studies of induced cartilage development and calcification. *J Cell Biol* 1967;35:81–101. [PubMed: 6061727]
 42. Bonucci E. Fine structure of early cartilage calcification. *J Ultrastruct Res* 1967;20:33–50. [PubMed: 4195919]
 43. Anderson HC. Calcific diseases. A concept. *Arch Pathol Lab Med* 1983;107:341–348. [PubMed: 6305305]
 44. Sela J, Schwartz Z, Amir D, Swain LD, Boyan BD. The effect of bone injury on extracellular matrix vesicle proliferation and mineral formation. *Bone Miner* 1992;17:163–167. [PubMed: 1611303]

45. Bellows CG, Aubin JE, Heersche JN, Antosz ME. Mineralized bone nodules formed in vitro from enzymatically released rat calvaria cell populations. *Calcif Tissue Int* 1986;38:143–154. [PubMed: 3085892]
46. Lowe J, Bab I, Stein H, Sela J. Primary calcification in remodeling haversian systems following tibial fracture in rats. *Clin Orthop Relat Res* 1983;176:291–297. [PubMed: 6851337]
47. Aaron JE, Oliver B, Clarke N, Carter DH. Calcified microspheres as biological entities and their isolation from bone. *Histochem J* 1999;31:455–470. [PubMed: 10475573]
48. Gorski JP, Wang A, Lovitch D, Law D, Powell K, Midura RJ. Extracellular bone acidic glycoprotein-75 defines condensed mesenchyme regions to be mineralized and localizes with bone sialoprotein during intramembranous bone formation. *J Biol Chem* 2004;279:25455–25463. [PubMed: 15004029]
49. McKee MD, Farach-Carson MC, Butler WT, Hauschka PV, Nanci A. Ultrastructural immunolocalization of noncollagenous (osteopontin and osteocalcin) and plasma (albumin and alpha 2HS-glycoprotein) proteins in rat bone. *J Bone Miner Res* 1993;8:485–496. [PubMed: 8475798]
50. Kajander EO, Ciftcioglu N. Nanobacteria: an alternative mechanism for pathogenic intra- and extracellular calcification and stone formation. *Proc Natl Acad Sci USA* 1998;95:8274–8279. [PubMed: 9653177]
51. Cisar JO, Xu DQ, Thompson J, Swaim W, Hu L, Kopecko DJ. An alternative interpretation of nanobacteria-induced biomineralization. *Proc Natl Acad Sci USA* 2000;97:11511–11515. [PubMed: 11027350]

Fig. 1.

Time course for mineral formation by MLO-A5 cells as detected using von Kossa stain. MLO-A5 cells were cultured with β GP and ascorbic acid as described previously [23]. After 3 days, cells become confluent; at 6 days, a “fractal” or “honeycomb” pattern was observed that continued to increase in staining until the mineral covered the entire well by 12 days. This process is delayed approximately 3 days in the absence of β GP (data not shown) [23]. The honeycomb pattern of mineralization is different from nodules formed by FRC cells and the ones formed by the osteoblast cell line 2T3, which require BMP-2 for mineralization to occur [32]. Images provided by Dr. Sarah Dallas (FRC nodules) and Wuchen Yang (2T3 nodules) in the laboratory of Dr. Steve Harris, University of Missouri at Kansas City.

Fig. 2. Visualization and quantitation of mineral formation by MLO-A5 cells using AR-S staining. A significant amount of staining is observed using either quantification by area or quantification by solubility. ## Significantly different from days 0, 3 and 6; ++ significantly different from day 0, 3, 6 and 9; ** significantly different from all other groups, all using oneway ANOVA followed by Tukey post test, $P < 0.001$.

Fig. 3. SEM showing the mineralized honeycombed matrix formed by MLO-A5 cells at day 9. Comparative secondary (**A, B**) and backscatter (**C**) images. Magnification is $\times 300$ (**A**) $\times 1,000$ (**B, C**). The cells appear to be sending (or leaving) projections along the mineralizing structures (*arrow*).

Fig. 4.

Elemental analysis of the mineralized “honeycomb” matrix formed by MLO-A5 cells at 12 days of culture. The calcium component (A) forms a pattern that overlays the phosphate component (C), which is consistent with the SEM image (B). EDS analysis shows a ratio of calcium to phosphorus that is similar to normal hydroxyapatite (D).

Fig. 5.

Western blot showing that the osteocyte-specific antigen E11 is increased at 6 and 9 days of culture, suggesting that these cells are becoming more osteocyte-like. Ponceau S stain shows relative amounts of loaded protein. MLO-Y4 cells are shown as a positive control.

Fig. 6.

Immunofluorescent staining for collagen type I expression in MLO-A5 cells. At day 0, when the cells become confluent, expression is only observed intracellularly, inside the cytoplasm of MLO-A5 cells. At 3 days, collagen is secreted into the ECM, forming bundles that continue to form through 6–9 days. At 12 days, the collagen appears more localized to the cytoplasm, again suggesting a layer of cells growing over the previously mineralized matrix.

Fig. 7. Visualization of collagen type I fibrils in MLO-A5 cells using TEM (A), AFM (B), and SEM (C, D). The collagen type I fibrils in these cultures show the characteristic 69 nm banding. Mineralized collagen fibrils are shown in D.

Fig. 8. Mineral is associated with collagen as shown by TEM of MLO-A5 cells at 12 days in culture. Magnification is $\times 3,000$ (**A**) and $\times 35,000$ (**B**) of the same section. At the same time, mineralized spheres of 100–250 nm (arrows) are associated with the cellular processes of MLO-A5 cells at 6 days of culture as determined by TEM (**C**).

Fig. 9.

The spheres contain calcium and phosphorus whether associated with the cell membrane (day 6) or with collagen fibers (day 9). At day 0, no spheres (arrows) are observed in confluent cells. By 6 days they are highly associated with cellular processes and mineralized. By day 9, they are also associated with collagen fibers. Elemental analysis using EDS shows that these structures contain both calcium and phosphorus, whether associated with the cell process or with collagen. Note the budding spheres (arrows) in the magnified area from day 6 cultures. The upper budding structure appears to be mineralizing, as indicated by the white on the tip of the bud.

◆, areas not containing spheres;

★, areas over spheres.

Fig. 10.

Comparative secondary and backscatter (**A, B**) images by SEM of cultures at day 6 showing the association of spheres with collagen fibrils at 6 days and still some association with cellular processes. Magnification is $\times 2,000$ (**A, B**). Note the spherical structure that appears to be budding (*arrow*) from the cell process in **A**. Note other spherical structures under the cell process, which are only visible with backscatter imaging (*arrows*) in **B**. (**C**) A sphere that appears to be engulfing collagen fibrils (9 days). These spheres appear to increase in size, engulf collagen fibers, and coalesce to form larger mineralized structures (*arrow*) as shown in 15-day culture (**D**). The sizes of these structures range from 50 nm to 1 μm .

Fig. 11. AFM height map of MLO-A5 cells at 12 days of culture showing spherical structures intercalated between collagen (**A**). The sizes of the spherical structures are 50 nm to 1 μ m, consistent with those in Figure 10 (**B**).

Fig. 12. SEM of FRC cells at 20 days of culture. Note in **A** that the mineralized spheres are only associated with areas of collagen production. A magnified image of the collagen fibrils, spheres, and potentially budding spheres is shown (**B**).

Fig. 13. TEM of osteoid-osteocytes in murine long bone. Note the small mineralized spheres in the osteoid, which appear to increase in size, coalesce, and eventually surround the cell (**A**). These mineralized structures are similar in size to those observed in MLO-A5 cells (*arrowhead*) and appear to be associated with dendritic processes (*arrow*) (**B**).

Fig. 14.

SEM of the ontogeny of osteoid-osteocytes in murine lamellar bone. (A) A cell newly embedding in osteoid. Dendritic processes extend toward the mineralization front. The front contains mineralized structures of similar size to the mineralized spheres observed in MLO-A5 and appear to increase in size. (B, C) Osteoid-osteocytes beginning to be surrounded by mineral. (D) Fully embedded osteocyte surrounded by mineral and a cell partially surrounded by mineral.

Fig. 15.

Our hypothesis is that as the osteoid-osteocyte becomes embedded in the nonmineralized matrix and converts from a polygonal matrix-producing osteoblast to a dendritic osteocyte, it begins to generate branching processes. As it forms these processes, small calcified spheres are formed along the cell membrane toward the mineralization front that eventually become associated with the collagen fibers that are simultaneously being produced by the cell. This occurs as the cytoplasm of the cells is shrinking and forming thinner and thinner processes. Therefore, a gradient in size of the mineralized spheres is observed. We propose that the osteoid-osteocyte initiates this gradient.