Eliminating Exposure to Aqueous Solvents Is Necessary for the Early Detection and Ultrastructural Elemental Analysis of Sites of Calcium and Phosphorus Enrichment in Mineralizing UMR106-01 Osteoblastic Cultures

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Key Words

Vesicle • Biomineralization • Osteoblast • Electron spectroscopic imaging • High-pressure freezing

Abstract

The mechanism underlying the mineralization of bone is well studied and yet it remains controversial. Inherent difficulties of imaging mineralized tissues and the aqueous solubility of calcium and phosphate, the 2 ions which combine to form bone mineral crystals, limit current analyses of labile diffusible, amorphous, and crystalline intermediates by electron microscopy. To improve the retention of calcium and phosphorus, we developed a pseudo nonaqueous processing approach and used it to characterize biomineralization foci, extracellular sites of hydroxyapatite deposition in osteoblastic cell cultures. Since mineralization of UMR106-01 osteoblasts is temporally synchronized and begins 78 h after plating, we used these cultures to evaluate the effectiveness of our method when applied to cells just prior to the formation of the first mineral crystals. Our approach combines for the first time 3 well-established methods with a fourth one, i.e. dry ultrathin sectioning. Dry ultrathin sectioning with an oscillating diamond knife was used to produce electron spectroscopic images of mineralized biomineralization foci

which were high-pressure frozen and freeze substituted. For comparison, cultures were also treated with conventional processing and wet sectioning. The results show that only the use of pseudo nonaqueous processing was able to detect extracellular sites of early calcium and phosphorus enrichment at 76 h, several hours prior to detection of mineral crystals within biomineralization foci.

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Introduction

Like cells in bone, osteoblastic cells in culture deposit mineral crystals extracellularly within multicellular nodules or focal biomineralization foci (BMF) [Quarles et al., 1992; Birnbaum and Wiren, 1994; Wang et al., 2000; Midura et al., 2004]. While results with primary osteo-

Abbreviations used in this paper

BGP	β-Glycerol phosphate
BMF	biomineralization foci
ESI	electron spectroscopic imaging

blastic cells and with the MC3T3-E1 cell line are similar, UMR106-01 osteoblastic cells have several features which facilitate analytical studies. Mineralization is rapid and occurs within 24 h after addition of a phosphate stimulus. Second, mineralization is temporally synchronized within a given culture and among different cultures so that each BMF progresses at the same rate [Wang et al., 2009]. Third, the amount of mineral deposited at 24 h within BMF is about 10-fold greater than that for MC3T3-E1 or primary calvarial osteoblastic cultures [Hale et al., 2000].

Electron spectroscopic imaging (ESI) or electron energy loss spectroscopic imaging is a sensitive analytical electron microscopic method with a spatial resolution of 0.5 nm and a mass resolution of <50 atoms [Simon, 1987]. It was developed by Ottensmeyer [1982] to image calcium, sulfate, and/or phosphorus atoms in calcifying cartilage and bone [Arsenault and Ottensmeyer, 1983]. This method has subsequently been used to characterize the calcification of dentine [Plate et al., 1992] as well as the mineralization of osteoblastic cells and chondrocytes in culture [Bordat et al., 1998; Rohde and Mayer, 2007]. ESI has not achieved the prominence it was initially projected to reach; this is in part because of the inherent difficulties associated with the routine application of high-pressure freezing and pseudo nonaqueous processing of specimens and a lack of methods to independently validate the resultant elemental distribution maps.

We show here that a combination of high-pressure freezing [Moor, 1987; Studer et al., 1989, 1995], freeze substitution [Van Harreveld and Crowell, 1964], ESI [Ottensmeyer, 1982], and dry ultrathin sectioning using an oscillating knife [Studer and Gnaegi, 2000] is necessary to detect focal extracellular areas of calcium and phosphorus enrichment several hours prior to the detection of crystalline mineral within BMF in the temporally synchronized UMR106-01 culture system.

Materials and Methods

Electron microscopy supplies were purchased at Plano GmbH (Wetzlar, Germany). UMR106-01 cells were kindly provided by Dr. R.J. Midura Jr. of the Cleveland Clinic and Foundation.

UMR106-01 Osteoblastic Cell Model

UMR106-01 cells were grown as previously described [Huffman et al., 2007]. For electron microscopy, cells were grown on sapphire discs (1.4 mm diameter; Leica microsystems, Vienna, Austria). Discs were first coated with a 10-nm carbon layer and then adhered to the bottom of sterile petri dishes by drying from a droplet of 5 µg/ml fibronectin in HEPES-buffered saline solution with the carbon side up. Cells (5×10^5 cells/ml) were added

to the petri dishes and allowed to adhere for 24 h to the coated sapphire discs; growth medium containing 10% FBS was then exchanged for medium containing 0.5% BSA (Millipore). Mineralization was induced at 64 h by the addition of fresh medium containing 6.5 mM β -glycerol phosphate (BGP). Mineral crystals began to form 14 h later (78 h after plating) [Wang et al., 2009]. Control cultures did not receive BGP and did not form mineral crystals. Cultures were cryo-fixed by high-pressure freezing 76 h after plating and processed for electron microscopic analysis as described below.

Conventional Chemical Fixation

The culture medium was removed and the cells on sapphire discs were immediately fixed in fresh 2.5% glutaraldehyde in 0.1 M cacodylate buffer. After 2 h, the cells were first washed with 0.1 M cacodylate buffer and then postfixated with 1% osmium tetroxide in 0.1 M cacodylate buffer. After rinsing with cacodylate buffer, the cells were dehydrated in a graded series of ethanol solutions and embedded in Epon/Araldite as described below.

High-Pressure Freezing and Freeze Substitution

Sapphire discs with attached cells were introduced (cell side up) into the cavity of membrane carriers (Leica microsystems). These platelets were mounted onto the pods with the help of the Leica transfer tool. The pods were then attached to a manipulator and high-pressure frozen (cooling rate approx. 20,000 K/s; pressure 2,040 bar) [Studer et al., 2001].

Platelets containing the frozen cells were transferred in liquid nitrogen to the freeze-substitution apparatus (AFS; Leica microsystems) where flow-through rings (Leica microsystems) were filled with precooled medium [acetone (dried over calcium chloride) containing 2% osmium tetroxide, 0.1% uranyl acetate, and 0.12% water] [Walther and Ziegler, 2002]. The AFS unit was programmed as follows: 30 h at -90°C , heating at a rate of $2^\circ\text{C}/\text{h}$ to -60°C , 8 h at -60°C , heating at a rate of $2^\circ\text{C}/\text{h}$ to -30°C , 24 h at -30°C , and transfer of the samples to ice (0°C) for 1 h. The specimens were then washed 3 times in anhydrous acetone and the frozen/freeze-substituted discs with cells were embedded at room temperature stepwise in Epon/Araldite (30, 70, and 100% acetone/resin). Infiltration times were 7 h for the first embedding step, 16 h for the second, and 1 day for the final resin concentration. Polymerization was then carried out with fresh resin at 60°C for 5 days.

Ultramicrotomy

The platelets were removed from the sample blocks by grinding. The sapphire discs were removed by repetitively submerging the embedded samples in liquid nitrogen to cool them down and then letting them warm up to room temperature (this cycle of freezing and warming was repeated until the sapphire disc detached from the Epon/Araldite). The samples were then reembedded to orient the cutting plane perpendicular to the plane of the cell layer, and they were subjected to ultramicrotomy with an Ultracut UCT (Leica microsystems). BMF were localized in semithin sections prior to ultrathin sectioning.

Wet sectioning was done with conventional diamond knives for transmission electron microscopy; sections were then post-stained prior to inspection with uranyl-acetate and lead citrate [Ellis, 2007]. Since wet sectioning was found to extract calcium and phosphorus, dry ultrathin sections (50 nm in thickness) were

Fig. 1. Confocal Raman spectral analysis detects protein changes preceding and accompanying mineralization within BMF. **a** Representative Raman confocal optical images of pre-BMF and BMF structures at different times over 24 h following addition of BGP. UMR106-01 cells were cultured on glass, and BGP was added to induce mineralization at 64 h. Individual BMF structures (10–25 μm in diameter) (arrows) were probed with a $\times 100$ objective and laser beam. Mineral crystals are visible as white deposits at 82 and 88 h. The blue spot depicts the position scanned by the laser Raman beam. **b** Overlay of Raman spectroscopic scans as a function of time (stage) of mineralization. Mineral crystals begin to nucleate within BMF at 76–82 h; the arrow denotes the 959-cm^{-1} phosphate band representing hydroxyapatite. The spectral scans depicted are the average of 10 different BMF/conditions as shown in **a**. Spectral profiles from different mineralization times are offset to improve clarity and are plotted on the same scale. **c** Raman peak height ratios varied progressively during the mineralization of BMF. Raman peak height ratios for selected Raman band positions were calculated from spectra in **b** relative to the invariant $1,450\text{-cm}^{-1}$ peak as a function of time after addition of BGP at 64 h. Data were analyzed by a 1-way ANOVA using multiple comparisons with application of the Holm-Sidak method. Asterisks represent values significantly different from the nonmineralized control at 64 h ($p < 0.05$). Error bars represent means $\pm$ SE (reproduced with permission from the Journal of Biological Chemistry).

produced from the freeze-substituted blocks using an oscillating diamond knife (Diatome, Biel, Switzerland) [Studer and Gnaegi, 2000]. The latter sections were not poststained to avoid contact with aqueous solutions.

Electron Microscopy

For electron energy loss analysis a LEO 912 with an omega-energy filter (Zeiss, Oberkochen, Germany) or a Tecnai F20 (FEI,

Eindhoven, The Netherlands) equipped with a Gatan GIF energy filter (Gatan, Munich, Germany) were used to analyze the sections. Electron spectroscopic imaging was used to qualitatively show the distribution of calcium and phosphorus in the images of interest. Calcium and phosphorus were detected at their L2 and L3 peaks (energy loss 343–354 eV and 132–153 eV, respectively) with the 3-window method (using a slit with an energy window of about 10 eV).

Fig. 2. Appearance of BMF after conventional chemical fixation of mineralizing osteoblastic cultures and wet sectioning. UMR106-01 cells were cultured for 12 h (76 h after plating) in the presence of BGP. **a** Parts of 2 cells and 2 BMF are depicted which contain darkly staining vesicles and heterogeneous matrix material whose structures cannot be clearly defined even at a higher magnification shown in **b**. Moreover, 1 BMF (**inset**) contains a poorly contrasted region containing a rather homogeneous, lightly stained organic matrix (arrowheads denote the edge of a poorly contrasted region).

Results

The process of mineralization of extracellular BMF in UMR106-01 cultures, which takes only 24 h to complete, is temporally synchronized [Gorski et al., 2004; Midura et al., 2004; Wang et al., 2009]. Based on confocal Raman spectroscopic analyses of the ν_1 symmetric stretch signal for phosphate (960 cm^{-1}) as a function of time, these cells produce a poorly crystalline form of hydroxyapatite which is first detectable ~ 78 h after plating (fig. 1). Optical images of the Raman scans showed that the calcium signal was punctuate in nature, reflecting the vesicular character of the phosphate content (fig. 1a). In order to evaluate the effectiveness of our pseudo nonaqueous method, we chose to stop the cultures at 76 h, 12 h after adding the BGP mineralization stimulus but about 2 h before the appearance of the first crystals of mineral (fig. 1) [Midura et al., 2004; Wang et al., 2009]. In this way, our goal was to identify the distribution pattern of labile calcium and phosphorus populations within BMF shortly before the nucleation of the first detectable mineral crystals.

Comparison of High-Pressure Frozen, Freeze-Substituted, and Conventionally Fixed Samples

All UMR106-01 cultures were grown for 76 h after plating, with the final 12 h in the presence of mineralization inducer BGP. Subsequently, some cultures were ran-

domly chosen to be processed for conventional fixation (fig. 2a, b), while others were processed by high-pressure freezing and freeze substitution (fig. 3a, b). Comparison of these paired high- and low-power images supports several conclusions. First, the conventionally fixed cultures resemble similar images published previously [Midura et al., 2004]. In all cases, extracellular BMF are composed of a heterogeneous collection of vesicles ranging in size from about 100 to 1,000 nm and displaying densities reflecting apparent differences in their contents (fig. 1b, 2a). Second, large areas are unstained and extend for over $5\text{ }\mu\text{m}$ in their longest dimension. One such area was partially captured in an inset (fig. 2a) and is enlarged in figure 2b (arrowheads). These areas exclude vesicles and other materials from their volume. Third, BMF from high-pressure frozen, freeze-substituted cultures display a dramatically different appearance. In contrast to large unstained regions, high-pressure frozen samples show 0.5- to $1\text{-}\mu\text{m}$ extracellular areas localized within BMF which are almost 'white' (arrow; fig. 3a). In the higher-power view, it is evident that despite the low contrast these 'white' regions do possess an underlying detail which represents a range of irregularly shaped spherical bodies ranging from about 50 to 800 nm in diameter (fig. 3b). However, these 'white' areas raised immediate concerns regarding the loss of inorganic and/or organic substances. In particular, when compared with similar BMF regions from paired conventionally fixed cultures

Fig. 3. High-pressure frozen, freeze-substituted osteoblastic cultures contain BMF with translucent spots which are poorly contrasted extracellular matrix regions. **a** UMR106-01 cells were grown identically to those in figure 2, but they were processed by high-pressure freezing and freeze substitution instead of conventional fixation. **b** The area within the inset in **a** was enlarged to illustrate that translucent spots possess an apparent substructure. When compared with analogous samples subjected to dry sectioning instead of wet sectioning, the translucent spots appear black (see fig. 4). As a result, we conclude that these extracellular regions of enrichment of calcium and phosphorus within BMF in **a** (arrow) were initially stained, but subsequently these elements and the associated stains were lost during the wet sectioning step.

(fig. 2), we hypothesized that ‘white’ spots represented materials which were preferentially retained by high-pressure freezing but which were subsequently lost during processing. Calcium and phosphorus detection in 76-hour-old cell cultures was not possible by ESI in either conventionally fixed or freeze-substituted samples which were sectioned on water regardless of whether specimens were poststained or not (results not shown). We therefore evaluated the impact of substituting dry sectioning for wet sectioning of high-pressure frozen, freeze-substituted cultures.

Electron Spectroscopic Imaging of Calcium and Phosphorus in High-Pressure Frozen, Freeze-Substituted Cultures Which Were Also Dry Sectioned

UMR106-01 cultures were again grown as described in Methods and induced to mineralize as described above. For comparison, BGP was omitted from controls which did not mineralize [Huffman et al., 2007]. All cultures were stopped at 76 h and processed by high-pressure freezing, freeze substitution, and dry sectioning. By comparison with the images in figure 3 which used wet sectioning, it is clear that substitution of dry sectioning leads to a dramatic increase in the retention of calcium and phosphorus within BMF (fig. 4). Focal 0.5- to 1-µm diameter areas which appeared as ‘white’ spots in figure 3 after wet sectioning appear dark (fig. 4a) in the zero loss energy image. The fact that UMR106-01 cultures miner-

alize in a reproducible, temporally synchronous manner facilitates these direct comparisons among different cultures [Wang et al., 2009]. In addition, ESI imaging demonstrates that the dark appearing areas are enriched in calcium and phosphorus (compare fig. 4a–c). Finally, as shown in the overlay image in figure 4d, the calcium (red) and phosphorus (green) signals largely overlap each other in the 76-hour cultures as shown by the presence of yellow. Since other studies have shown that 76-hour UMR106-01 cultures do not contain detectable mineral crystals [Huffman et al., 2007; Wang et al., 2009], the enriched contents of calcium and phosphorus observed here could represent amorphous calcium phosphate [Driessens et al., 1978] and/or labile organic forms of phosphorus such as polyphosphates [Omelson et al., 2009]. Interestingly, a functional role for the observed enriched focal contents of calcium and phosphorus in mineralization is supported by analyses of unmineralized control cultures. When a similar high-pressure freezing, freeze substitution, and dry sectioning approach was applied to unmineralized UMR106-01 cultures, few dark appearing vesicles or particles were detected (fig. 5). Those dark areas which were observed contained little phosphate (fig. 5b, d). Finally, an additional advantage of using the oscillating knife was that it reduced compression during sectioning and it reduced wrinkling of the resultant sections.

Fig. 4. After the pseudo nonaqueous processing method, mineralized BMF contained areas enriched in phosphorus and calcium. UMR106-01 cells were grown identically to those in figure 2. At 76 h, the cultures were stopped and processed by high-pressure freezing, freeze substitution, and dry sectioning. **a** Zero loss energy image depicting numerous dark appearing vesicles or particles. **b** Electron spectroscopic imaging of the calcium signal within dark appearing vesicles or particles. **c** Electron spectroscopic imaging of phosphorus signal within dark appearing vesicles or particles. **d** Superimposed images of the views in **a–c**.

Fig. 5. Control, unmineralized BMF contain few focal enriched areas of calcium and phosphorus content. UMR106-01 cells were grown identically to those in figure 2 except that mineralization was blocked by omission of BGP. At 76 h, the cultures were stopped and processed by high-pressure freezing, freeze substitution, and dry sectioning. **a** The zero loss energy image of an unmineralized BMF contains few darkly stained vesicles or particles (only 2 dark spots in this field). **b** Electron spectroscopic imaging of the darkly stained vesicles or particles yields a negative calcium signal. **c** Electron spectroscopic imaging of the darkly stained vesicle or particle yields negative phosphorus signal. **d** Superimposed images of the views in **a–c**.

Discussion

The data presented in this paper support the following conclusions about the mineralization of BMF within temporally synchronized UMR106-01 osteoblastic cultures. High-pressure freezing and pseudo nonaqueous processing are required to detect extracellular sites of early calcium and phosphorus enrichment at 76 h, several hours prior to the formation of mineral crystals within BMF. In particular, use of dry sectioning proved to be a key step in the preservation of calcium and phosphorus. Also, use of electron spectroscopic imaging demonstrated that darkly stained vesicles within extracellular BMF are enriched in calcium and phosphorus prior to the detection of crystalline mineral forms.

Our findings are consistent with previous studies on calcifying cartilage and tendon and intramembranous bone and periosteal bone [Arsenault and Ottensmeyer, 1983, 1984]. In those studies epiphyseal growth plates revealed a developmental sequence leading from a noncalcified matrix to a calcified matrix wherein, in the initial stages, matrix granules enriched predominantly in P were distributed separately from Ca-containing sites. At a later stage of mineralization, both calcium and phosphorus were found to instead colocalize with irregularly shaped mineral deposits in the zone of provisional calcification [Arsenault and Ottensmeyer, 1983]. Interestingly, Arsenault and Ottensmeyer [1983] also detected initial elevated focal concentrations of phosphorus in the early

stages of intramembranous bone mineralization which were separate from those of calcium [Arsenault and Ottensmeyer, 1984].

Other studies have previously shown that additional methods (high-pressure freezing and freeze substitution) are needed to avoid loss during specimen fixation, embedding, and sectioning [Höhling and Krefting, 1984; Arsenault and Hunziker, 1988; Landis and Hodgens, 1990; Keene and McDonald, 1993; Engfeldt et al., 1994]. However, most prior studies have not consistently avoided water during specimen processing since it lowers image contrast due to the limited solubility of stains in organic solvents and it necessitates dry sectioning of blocks. Thus, the true impact of pseudo nonaqueous processing on the mechanism of osteoblast-mediated mineralization is difficult to assess from a consideration of the published literature. Our results clearly demonstrate the advantage of the high-pressure freezing and nonaqueous processing approach in preserving and detecting diffusible forms of calcium and phosphorus during mineralization of BMF in osteoblastic cell cultures.

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