Bone 71 (2015) 244–256

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Original Full Length Article

Extracellular matrix mineralization in murine MC3T3-E1 osteoblast cultures: An ultrastructural, compositional and comparative analysis with mouse bone

W.N. Addison [a,1,2], V. Nelea [a,1], F. Chicatun [b], Y.-C. Chien [a,3], N. Tran-Khanh [c], M.D. Buschmann [c], S.N. Nazhat [b], M.T. Kaartinen [a], H. Vali [d], M.M. Tecklenburg [e], R.T. Franceschi [f], M.D. McKee [a,d,*]

[a] Faculty of Dentistry, McGill University, Montreal, Quebec, Canada
[b] Department of Mining and Materials, McGill University, Montreal, Quebec, Canada
[c] Department of Chemical Engineering, École Polytechnique, Montreal, Quebec, Canada
[d] Department of Anatomy and Cell Biology, McGill University, Montreal, Quebec, Canada
[e] Department of Chemistry, Central Michigan University, Mount Pleasant, MI, USA
[f] Department of Periodontics and Oral Medicine, School of Dentistry, University of Michigan, Ann Arbor, MI, USA

ARTICLE INFO

Article history:
Received 11 August 2014
Revised 30 October 2014
Accepted 6 November 2014
Available online 13 November 2014

Edited by: Mark Johnson

Keywords:
Cell culture
Biomineralization
Hydroxyapatite
Osteoblasts
MC3T3-E1 cells
Extracellular matrix

ABSTRACT

Bone cell culture systems are essential tools for the study of the molecular mechanisms regulating extracellular matrix mineralization. MC3T3-E1 osteoblast cell cultures are the most commonly used *in vitro* model of bone matrix mineralization. Despite the widespread use of this cell line to study biomineralization, there is as yet no systematic characterization of the mineral phase produced in these cultures. Here we provide a comprehensive, multi-technique biophysical characterization of this cell culture mineral and extracellular matrix, and compare it to mouse bone and synthetic apatite mineral standards, to determine the suitability of MC3T3-E1 cultures for biomineralization studies. Elemental compositional analysis by energy-dispersive X-ray spectroscopy (EDS) showed calcium and phosphorus, and trace amounts of sodium and magnesium, in both biological samples. X-ray diffraction (XRD) on resin-embedded intact cultures demonstrated that similar to 1-month-old mouse bone, apatite crystals grew with preferential orientations along the (100), (101) and (111) mineral planes indicative of guided biogenic growth as opposed to dystrophic calcification. XRD of crystals isolated from the cultures revealed that the mineral phase was poorly crystalline hydroxyapatite with 10 to 20 nm-sized nanocrystallites. Consistent with the XRD observations, electron diffraction patterns indicated that culture mineral had low crystallinity typical of biological apatites. Fourier-transform infrared spectroscopy (FTIR) confirmed apatitic carbonate and phosphate within the biological samples. With all techniques utilized, cell culture mineral and mouse bone mineral were remarkably similar. Scanning (SEM) and transmission (TEM) electron microscopy showed that the cultures had a dense fibrillar collagen matrix with small, 100 nm-sized, collagen fibril-associated mineralization foci which coalesced to form larger mineral aggregates, and where mineralized sites showed the accumulation of the mineral-binding protein osteopontin. Light microscopy, confocal microscopy and three-dimensional reconstructions showed that some cells had dendritic processes and became embedded within the mineral in an osteocyte-like manner. In conclusion, we have documented characteristics of the mineral and matrix phases of MC3T3-E1 osteoblast cultures, and have determined that the structural and compositional properties of the mineral are highly similar to that of mouse bone.

Introduction

Bone cell culture systems are essential tools for the investigation of the molecular mechanisms that regulate mineralization of the extracellular matrix (ECM) [1]. Such studies provide fundamental information on biomineralization important to understanding basic skeletal (and dental) biology, and to managing mineralization-related pathologies such as the osteomalacias and ectopic calcifications. Data on mineralization mechanisms obtained from cell culture models are also valuable for the rational design of bone repair strategies and tissue engineering

* Corresponding author at: Faculty of Dentistry, Room M73, McGill University, 3640 University Street, Montreal, Quebec H3A 0C7, Canada. Fax: +1 514 398 8900.
E-mail address: marc.mckee@mcgill.ca (M.D. McKee).
[1] These authors contributed equally to this manuscript.
[2] Present address: Department of Oral Medicine, Infection and Immunity, Harvard School of Dental Medicine, Boston, MA 02115, USA.
[3] Present address: Department of Preventive and Restorative Dental Sciences, University of California San Francisco, San Francisco, CA 94143-0758, USA.

http://dx.doi.org/10.1016/j.bone.2014.11.003
8756-3282/

constructs, and biomaterial innovation. For *in vitro* studies to have appropriate relevance, it is important that the mineralization process and the mineral and matrix formed be physiologic in nature.

Although there are a multitude of material characterization techniques potentially applicable to the study of biomineral deposited *in vitro* (and also *in vivo*), there is little work in the literature describing a systematic characterization of the biomineral formed in osteoblast cell cultures. Most investigators rely initially on classical histologic methods using von Kossa or Alizarin Red staining, even though these stains have repeatedly been shown to be insufficient for the accurate assessment of the mineral phase generated in cell cultures [2]. Limitations of such assays rest on the inherent nonspecific staining of these chemical reagents. For example, although the von Kossa reagent (silver nitrate) stains for phosphate, it does not specifically stain apatitic mineral and may stain other mineral phases, just as it may potentially stain high concentrations of protein-bound phosphate in bone [3]. Likewise, Alizarin Red chelates calcium and will stain calcium regardless of its source, potentially including extracellular matrix mineral, salts, precipitates, protein-bound calcium or even free calcium in solution [4]. Moreover, these same chemical stains will stain nonphysiologic dystrophic calcification [2].

Bones require for their function not only an appropriate mineral phase, but also mineral having correct crystallite shape and size, orientation, location and quantity, and an appropriate crystallinity (that includes lattice substitutions), all occurring within a dense, mature collagenous matrix that also contains noncollagenous proteins and small proteoglycans. MC3T3-E1 cells are a murine calvaria-derived pre-osteoblastic cell line used as an archetypal model of *in vitro* osteogenesis [5], being the most commonly used cell line in *in vitro* bone cell biology research. Following isolation and cloning of the MC3T3-E1 cell line on the basis of high alkaline phosphatase activity (an early marker of osteoblast activity), MC3T3-E1 cells have been further subcloned based on their potential to mineralize and on their expression of late markers of osteoblast differentiation such as osteocalcin and bone sialoprotein [6].

Despite the widespread use of the MC3T3-E1 cell line as a model of osteogenesis, the mechanism by which the extracellular matrix mineralizes remains unclear, and to date there is no detailed systematic characterization of the mineral deposited in these cultures. To re-evaluate and definitively determine the suitability of MC3T3-E1 osteoblast cultures as a model for investigating molecular determinants of bone mineralization, we performed a multi-technique, biophysical and ultrastructural assessment of the mineral and matrix–mineral relationships and architecture in these cultures. This work builds upon previous studies that have used similar material characterization techniques as those we have applied here on mineral deposited in cell cultures [7–9].

Our present comprehensive characterization of the morphology, composition and structure of the biomineral deposited by MC3T3-E1 osteoblasts *in vitro*, and its comparison to bone mineral and synthetic mineral standards, provides insight into potentially important similarities and differences between bone mineral formed *in vivo versus* that formed *in vitro*. The work also establishes parameters for MC3T3-E1 culture use in biomineralization studies. In addition, it improves our understanding of the relationship between the organic extracellular matrix and the biogenic inorganic mineral phase at the ultrastructural level. Taken together, this study addresses the suitability and limitations of a bone cell culture system for investigating mineralization processes, and it provides detailed mineral information on the MC3T3-E1 cell culture model justifying its continued use in mechanistic bone mineralization studies.

Materials and methods

Cell culture conditions

MC3T3-E1 murine calvarial osteoblasts (subclone 14) [6] were maintained in modified minimum essential medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Hyclone, Waltham, MA, USA) (selected from multiple lot/batch testing to be permissive of mineralization) and 1% penicillin–streptomycin (Invitrogen) at 37 °C in a humidified atmosphere of 5% CO_2. All experiments were carried out using cell culture dishes (Corning Inc., Corning, NY, USA) or wells (Sarstedt, Newton, NC, USA) at a plating density of 50,000 cells/cm^2. Cell differentiation and matrix mineralization were initiated 24 h after plating, with medium supplemented with 50 μg/ml ascorbic acid (AA) (Sigma-Aldrich, St. Louis, MO, USA), and 10 mM β-glycerophosphate (Sigma-Aldrich) as a phosphate source enabling mineralization. Calcium was available at 1.8 mM as a constituent of the culture medium. The medium was changed every 48 h.

Mineral preparations

Mineral samples were prepared and analyzed in two forms, *i.e.* either as synthetic or pulverized/ground powders, or as resin-embedded intact samples.

Hydroxyapatite control standards

Carbonated (7.7% w/w) hydroxyapatite standard powder (C-HA) was prepared following the aqueous precipitation method reported by Penel et al. [10], except that ammonium salts were used (ammonium phosphate dibasic and ammonium carbonate). The carbonate level was determined by Fourier-transform infrared spectroscopy (FTIR) using a calibration set of carbonated apatites with carbonate content determined by elemental analysis. Hydroxyapatite standard reference material powder (HA-SRM) was from NIST (Standard Reference Material® 2910, National Institute of Standards and Technology, Bethesda, MD, USA).

Isolation of mineral crystals from MC3T3-E1 osteoblast cultures

Cell culture mineral crystals were isolated following a modified protocol described previously [11]. Briefly, cell culture plates from day-12 mineralized osteoblast cultures were gently rinsed three times with double-distilled water produced by the Simplicity Purification System (ddH_2O, pH 8.0, resistivity 18.2 MΩ cm, Millipore, Billerica, MA, USA) followed by gentle spatula scraping of the cell culture layer (including the extracellular matrix and mineral) from the dish surface, and air drying. Samples were pulverized/ground, and then incubated (or not incubated) in 1.3% sodium hypochlorite (NaOCl, Acros Organics, New Jersey, NJ, USA) to remove organic components, then sonicated briefly for several min. Crystals were pelleted by centrifugation and then immediately washed three times in ddH_2O before sequential dehydration in 50%, 95% and 100% ethanol (Fisher Scientific Co., Ottawa, ON, Canada). Crystals were stored in 100% ethanol at −20 °C.

Mouse calvarial bone

Calvarial bone from 1-month-old Balb/C mice were first rinsed briefly with physiologic saline and ddH_2O, air-dried, and then pulverized/ground extensively in an agate mortar. Specifically for powder X-ray diffraction (XRD, see below in Section X-ray diffraction), treated (with sodium hypochlorite, as above) inorganic bone samples were used because unusable, high-background noisy spectra were obtained from pulverized/ground untreated bone, and since the treatment has been shown not to alter the structure and composition of mineral crystals disaggregated from bone [11]. For this, ground bone (50–100 mg equivalent of 3 whole calvariae) was digested in 10 ml of 1.3% sodium hypochlorite at room temperature with continuous sonication for 10 min. Crystals were pelleted by centrifugation, and then immediately washed three times in ddH_2O and then sequentially in ddH_2O before dehydration in 50%, 95% and 100% ethanol. Crystals were stored in 100% ethanol at −20 °C.