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Characterization of primary osteocyte-like cells from rat mandibles

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Abstract

Objective—The mandible is continuously undergoing remodeling due to mechanobiological factors, such as chewing forces, tooth loss, orthodontic forces, and periodontitis. The effects of mechanical stress and biological signals in bone homeostasis have been the focus of many investigations. However, much of this research utilized osteocytes derived from long bones while little is known about the mandible-derived osteocytes. This study tests a protocol to isolate and grow osteocytes from rat mandible.

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CONFLICTS OF INTEREST

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Design—Rat mandibles were harvested, sectioned into small pieces and subjected to a sequence chemical treatment and enzymatic digestion. The treated tissues were cultured for a few weeks while cells emerged. Cells were sorted by using the osteocyte marker podoplanin, an early marker for osteocyte differentiation. The cells were then characterized according to morphology, biochemical markers (osteocalcin, podoplanin, and sclerostin) and alkaline phosphatase activity and compared to an isotype cell line MLO-Y4 cells.

Results—The mandibular osteocytic-cells had stellate shape and were positive for osteocalcin, podoplanin and sclerostin and low alkaline phosphatase activity compared to MLO-Y4 osteocyte-like cells.

Conclusions—The protocol to isolate osteocyte-like cells will allow the investigators to investigate the mechanobiological differences in biomechanical response between these mandibular and long-bone osteocyte-like cells under various conditions.

Keywords

Bone; mandible; osteocyte; isolation; cell-culture; primary; MLO-Y4

Introduction

The mandible has a complex geometry with two vertical rami bilaterally that articulate with the base of the skull and a U-shaped body that consists of two bone types, alveolar and basal bone.^{1, 2} The alveolar portion of the mandible serves to accommodate the teeth and their suspensory periodontal ligaments,³ the two structures that play a significant role in the mandibular resistance to mechanical stress.⁴ This complex mandibular design defines its strength and stiffness.^{1, 2} Severe alveolar bone loss in the mandible is often due to the extraction of teeth, periodontitis, or aging⁵. Bone loss occurs predominately in the alveolar bone, with very little resorption occurring in the basal bone⁶. The load exerted on the mandible during mastication is determined by multiple structures working simultaneously: the muscles of mastication, the temporomandibular joint and the teeth. Such stresses are difficult to measure accurately in vivo⁷, which makes the study of factors controlling bone resorption difficult⁸. The problem becomes even more complicated by considering any alteration of the normal mandibular anatomy after tooth extraction, implant placement and/or orthodontic treatment.⁸ The patterns and rates of alveolar bone resorption after dental extraction are variable for each jaw.^{5, 9, 10} The stress/strain patterns can also be influenced by FACTORS, such as gender and behavior of the subject, consistency and change of food material properties between chewing cycles.^{11–13}

Osteocytes represent 90–95% of the bone cell population.^{14, 15} Osteocyte number varies between species and it is inversely proportional to the animal size.¹⁶ Osteocyte density also varies according to bone type, the number being highest in rapidly forming and remodeling woven bone.¹⁷ Osteocytes are formed by the encasement of osteoblasts within the bone matrix creating a lacuno-canalicular network. This network is characterized by osteocytes maintaining intercellular connections via gap junctions to the neighboring cells as well as the bone lining cells and the extraosseous surface.^{18–20} The osteocytes are embedded in a matrix filled with proteoglycans that play a role in the amplification of fluid flow initiated by

mechanical signals.²¹ Despite the small volume of the lacuno/canalicular system fluid, it has very large surface area compared to the Haversian and Volkmann system as a whole.²² Osteocyte structure and organization allowing orchestration of bone response to the mechanical load.^{23,24} Studies that have elucidated the role of osteocytes in bone homeostasis were performed on long bone and long bone-derived osteocyte-like cells^{25, 26}.

Very little known about the jaw-derived osteocytes, as osteocytes are difficult to isolate because of their location inside hard matrix.²⁷ Many studies of osteocyte behavior were based on histological or immunohistochemical staining of bone sections.^{27, 28} Previous trials to isolate osteocyte-like cells have been carried out through sequential enzymatic digestions of calvarial bone.^{29,30} It was not until the development of antibodies specific to osteocyte membrane antigens (i.e.monoclonal antibody (Mab) OB 7.3 against avian osteocytes) that isolation and confirmation of isolated osteocytes was achieved.^{30, 31} These techniques have become useful in isolation and culture primary osteocytes to study bone health and disease. Although most studies used young animals to isolate osteocytes, the technique has been used to isolate osteocytes from mature and aged mice long bones.³²

One of the most commonly used osteocyte cell-lines is the MLO-Y4 osteocyte-like cells isolated from transgenic mice long bone.³³ These cells were isolated by targeting SV40 large T-antigen oncogene and were confirmed to be osteocytic cells by morphology, high osteocalcin and low alkaline phosphatase gene expression.³⁴ Additional osteocyte markers have been identified such as dentin matrix protein-1 (DMP-1) located on the osteocyte cell process and pericellular matrix^{35, 36} and podoplanin, also known as E11, secreted by naïve osteocytes during bone remodeling and fracture healing and responsible for formation of processes and osteocytes attachment to bone matrix.³³ Cell-lines are modified to allow for easier culture and homogeneous phenotype compared to primary cells. As such it is unknown if individual cell lines respond to stimulus similar to related primary cells.

This study establishes a protocol for the isolation of osteocytic-cells from rat mandibles. Our hypothesis is that these isolated primary cells will exhibit similar osteocyte-like characteristics as MLO-Y4 cells with stellate shape, positive staining for osteocalcin, podoplanin and sclerostin and low alkaline phosphatase activity. These cells will be utilized for biomechanical testing studies to determine the mechanism of the selective resorption of alveolar bone as well as disease states of the mandible. Since osteocyte is the cell type responsible for the mechanobiology of bone, there is a need to identify how mandibular osteocytes respond to changes in loading as compared to osteocytic cells from the long bone.

Materials and methods

Cell isolation and culture (Fig. 1)

The investigation conforms to the guide for the care and use of laboratory animals published by the US National Institutes of Health. The study was approved by Georgia Regents University Institutional Animal Care and Use Committee.

Six four weeks old male Sprague-Dawley rats were used to harvest cells. The animals were anesthetized by a ketamine/xylazine cocktail. Intraperitoneal injection with 0.2 ml of

Xylazine sterile solution 20 mg/ml (lloyd laboratories, Shenandoah, Iowa) and ketamine HCl 100 mg/ml (Bioniche Teoranta Inverin, Rosemont, IL). The animals were then euthanatized by cervical dislocation and thoracotomy and mandibles were harvested. Under a dissecting microscope, mandibles were saturated with Hanks Balanced Salt Solution (HBSS), soft tissue was scraped and teeth removed. Mandibles were then sectioned into 3 mm fragments using a scalpel. Fragments were saturated in 70 % ethyl alcohol for 15 seconds to lyse all superficial cells, rinsed twice with HBSS and Phosphate Buffered Saline (PBS). Bone fragments were then incubated at 37 °C with gentle agitation for 20 minutes in collagenase type A, (1 mg/ml; Invitrogen, NY, USA) dissolved in HEPES buffer, 40 mM, CaCl₂ 4.8 mM, L-Glutamine 1%, penstrep (Penicillin/Streptomycin) 2%, and 100 ml serum free Dulbecco's Modified Eagle Medium (DMEM)) for four incubations. The fragments were then washed with PBS three times and cut into smaller 1 mm pieces. The fragments then explanted into 100 ml petri dishes and cultured in DMEM media, 1% penstrep, 0.5 % amphotericin B, 1% L-glutamine (Fischer Scientific, PA, USA) and supplemented with 10 % fetal bovine serum (FBS) (Atlanta Biologicals, GA, USA). Explants were incubated at 37 °C and 5% CO₂ and kept undisturbed for 7–10 days before first media change. Cells from each animal were cultured independently from each other.

Cryopreserved MLO-Y4 cells (gift of Dr. Lynda Bonewald) were grown per investigator's instructions in DMEM medium supplemented with 2.5% fetal bovine serum, 2.5% calf serum, and 1% pen/strep.

Fluorescence-Activated Cell Sorting (FACS, Fig. 1C)

Cells were labeled with fluorescein isothiocyanate (FITC)-conjugated podoplanin, specific osteocyte surface marker (Biorbyt Ltd. Waterbeach, Cambridge, UK)³⁷, according to the standard of FACS analysis and manufacturer's instructions. Cell sorting was performed on a Beckman Coulter MoFlo Cell Sorter (Fort Collins, CO). Cells were selected by first gating on forward scatter (FSC) versus side scatter (SSC), then, after displaying the chosen FSC/SSC cells on the appropriate fluorescence histogram, positive/negative cells were chosen for sorting. This work was performed in and with the assistance of Georgia Regents University's campus flow cytometry core facility. The sorted cells were sub-cultured and characterized by immunohistochemistry (IHC), transmission electron microscopy (TEM for morphology) and alkaline phosphatase activity.

Immunohistochemistry

Isolated cells were cultured for 2 days on chamber slides washed with PBS and fixed with 3% paraformaldehyde in PBS for 15 minutes. The cells were permeabilized with 0.1 % Triton-X in PBS for 10 minutes, and incubated with 0.3 % H₂O₂ for 10 minutes at room temperature. Cells were blocked with secondary-host serum for 20 minutes and incubated with rabbit polyclonal antibodies specific for osteocalcin (1:250; Abbiotec, CA, USA), sclerostin (1:150; Abcam, MA, USA) and podoplanin (1:100; Abbiotic, CA, USA) for 90 minutes at room temperature. After washing the cells three times, cells were then incubated with secondary biotinylated horse universal antibody, ABC reagent and DAP (ABC kit, Vector Lab, CA, USA), respectively, according to manufacturer instructions. Cells were counterstained with hematoxylin for 1 minute, dehydrated and cover slipped. liver tissue was

used as positive control for osteocalcin and sclerostin white lunch tissue was used as positive controls for podoplanin (Fig 2). Negative controls were used by staining our isolated cells (Fig 3 A) and MLO-Y4 (Fig 3 B) without primary antibody to exclude non-specific signals and false positive results.

Transmission Electron Microscopy (TEM)

Cells from each animal were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate (NaCac) buffer, pH 7.4, post-fixed in 2% osmium tetroxide in NaCl, stained en bloc with 2% uranyl acetate, dehydrated with a graded ethanol series and embedded in epon-araldite resin. Thin sections were cut with a diamond knife on a Leica EM UC6 ultramicrotome (Leica Microsystems, Inc, Bannockburn, IL), collected on copper grids and stained with uranyl acetate and lead citrate. Cells were observed in a JEM 1230 transmission electron microscope (JEOL USA Inc., Peabody, MA) at 110 kV and imaged with an UltraScan 4000 CCD camera & First Light Digital Camera Controller (Gatan Inc., Pleasanton, CA). (Fig. 3, Table 1)

Alkaline phosphatase activity

Alkaline phosphatase activity was detected using an alkaline phosphatase colorimetric assay kit (Abcam, MA, USA) according to manufacturer instructions. The kit exploits the colorimetric change of p-nitrophenyl phosphate (PNPP) as alkaline phosphatase dephosphorylates the substrate. The results were measured spectrophotometrically at 750 nm using a Versamax micro plate reader (Molecular Devices CA, USA.). (Fig. 5)

Results

Isolation of osteocyte-like cells from mandibular bone is a difficult process because of the existence of these cells within the bone matrix. Multiple steps of bone cutting and sequential digestion process were done to treat mandibular bone fragments. The fragments were cultured in petri dishes (Fig 1A). After two weeks in culture, high number of viable primary osteocyte-like cells migrated out of the explanted bone fragments (Fig. 1 B). The cells showed stellate morphology characteristic of osteocytes. To determine the percentage of osteocytes obtained, a Fluorescence-Activated Cell Sorting was done using podoplanin osteocyte marker. The FACS showed that 91.58% were podoplanin-positive (sample sorting graph is shown in Fig 1C). Further characterization using more osteocyte specific markers revealed that the mandibular osteocytic-cells stained similarly positive for osteocalcin, podoplanin and sclerostin comparable to MLO-Y4 cells (Figure 2).

TEM micrographs revealed that the ISOLATED primary mandibular cells (Fig 4 D, E, F) had similar number of mitochondria, but fewer dilated rough endoplasmic reticulum COMPARED TO the MLO-Y4 cells (Fig 4A, B, C, Table 1).³⁸ The isolated mandibular cells yielded low alkaline phosphatase activity THAN THE MLO-Y4 osteocyte-like cells.

Discussion

Cells isolated from rat mandible exhibited the stellate morphology characteristic of osteocytes and were podoplanin (E11) positive. Podoplanin is one of the earliest osteocyte-

selective markers as the osteoblast is embedded and differentiates into an osteocyte. This protein first appears on the dentritic processes. It promotes dentritic process elongation, and is important for osteocyte cell activity³⁹. Podoplanin expression is strong evidence of an osteocyte-like phenotype. Morphologically the sorted cells continued to have stellate shape with long protrusions maintaining contact with each other throughout cell testing.

Further characterization revealed that the mandibular osteocytic-cells stained similarly for osteocalcin, podoplanin and sclerostin as MLO-Y4 cells. Osteocalcin is involved in the regulation of bone mineralization⁴⁰(39)(40)(41) and is highly expressed in osteocytes,³⁴ (33,34) Podoplanin expression was maintained during culturing after cell-sorting. Sclerostin which is expressed in mature osteocytes was shown to be similar in both our isolated cells and MLO-Y4.⁴¹

TEM micrographs revealed that the primary mandibular cells (Fig 3D, E, F) had similar number of mitochondria, but fewer dilated rough endoplasmic reticulum compared to MLO-Y4 cells (Fig 3A, B, C, Table 1). The isolated mandibular cells yielded low alkaline phosphatase activity compared to MLO-Y4 osteocyte-like cells which has a low response as well. This finding is consistent with their function. Osteocytes have low alkaline phosphatase activity response because they are entrapped and no longer mineralizing their matrix, while osteoblasts are typically highly responsive³⁴.

In summary, we propose a protocol for isolation of mandibular cells exhibited osteocyte-like morphology as well as tested positive for osteocytic biochemical markers osteocalcin, podoplanin (sorting and IHC) and sclerostin. Furthermore, the cells demonstrated low alkaline phosphatase activity. This profile supports the identity of these primary mandibular cells as being osteocyte-like cells. Osteoblasts from tibia and mandible have differences in proliferation, alkaline phosphatase activity and the bone growth regulators.⁴² These differences may be also expressed in osteocytes isolated from the same tissues. Therefore, using these isolated cells, we will investigate the mechanobiological differences in biomechanical response between these mandibular and long-bone osteocyte-like cells under various conditions and disease states including osteonecrosis of the jaw. The ability to investigate the role of osteocytes in mandibular mechanobiology, health, and disease will let to better treatment and patient outcomes. Although others have used similar techniques to isolate osteocytes, this laboratory is the first to our knowledge to culture mandibular osteocytes. This advancement will allow further studies targeting specific oral health issues. More investigation may be needed to verify the sustained purity of cell type using osteocyte and osteoblast marker during culturing to ensure dedifferentiation is not occurring.

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There is a need to identify how mandibular osteocytes respond to changes in loading as compared to osteocytic cells from the long bone. And also how alveolar osteocytes respond to change of loading as compared to basal bone osteocytes.

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Figure 1.
Culturing of osteocytes from mandibular bone.
A: Enzymatically digested bone explants in DMEM media.
B: Bone explants after 2–3 weeks showing one bone explants (*) and the cells emerging out.
C: FACS graph shows gating of E11 positively labeled cells
D.: Plated E11 positive cells expressing multiple interconnected processes similar to osteocytes (20× magnification)

Figure 2.

Immunohistochemistry slides of cells using osteocalcin, podoplanin, and sclerostin.

- A. Osteocalcin staining of mandibular cells
- B. Osteocalcin staining of MLO-Y4 cells
- C. Positive staining of osteocalcin control specimen
- D. Podoplanin staining of mandibular cells
- E. Podoplanin staining of MLO-Y4 cells
- F. Positive staining of podoplanin control specimen
- G. Sclerostin staining of mandibular cells
- H. Sclerostin staining of MLO-Y4 cells
- I. Positive staining of sclerostin control specimen

Figure 3.
Negative controls for isolated cells (A) and MLO-Y4 (B)

Figure 4. TEM micrographs of MLO-Y4 cells (upper, A, B, C) and primary mandibular osteocyte-like cells (lower, D, E, F). Magnification bars located in each micrograph.

Figure 5.
Alkaline phosphatase activity response of primary osteocyte-like cells and MLO-Y4 cells.
Asterisk represents significant difference $\alpha=0.05$

Table 1

TEM Morphological Characteristics

	Preosteoblast	Osteoblast	Osteocyte
Shape	Less cuboidal	Typically cuboidal	Stellate-shaped with more processes
Organelles		Rich in ER & Golgi Few mitochondria Large eccentric nucleus	Nascent osteocyte is similar to osteoblasts, while mature osteocytes have fewer ER, Golgi & Mitochondria