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A three-dimensional tissue culture model of bone formation utilizing rotational co-culture of human adult osteoblasts and osteoclasts

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ABSTRACT

Living bone is a complex, three-dimensional composite material consisting of numerous cell types spatially organized within a mineralized extracellular matrix. To date, mechanistic investigation of the complex cellular level cross-talk between the major bone-forming cells involved in the response of bone to mechanical and biochemical stimuli has been hindered by the lack of a suitable in vitro model that captures the "coupled" nature of this response. Using a novel rotational co-culture approach, we have generated large (>4 mm diameter), three-dimensional mineralized tissue constructs from a mixture of normal human primary osteoblast and osteoclast precursor cells without the need for any exogenous osteoconductive scaffolding material that might interfere with such cell–cell interactions. Mature, differentiated bone constructs consist of an outer region inhabited by osteoclasts and osteoblasts and a central region containing osteocytes encased in a self-assembled, porous mineralized extracellular matrix. Bone constructs exhibit morphological, mineral and biochemical features similar to remodeling human trabecular bone, including the expression of mRNA for SOST, BGLAP, ACP5, BMP-2, BMP-4 and BMP-7 within the construct and the secretion of BMP-2 protein into the medium. This "coupled" model of bone formation will allow the future investigation of various stimuli on the process of normal bone formation/remodeling as it relates to the cellular function of osteoblasts, osteoclasts and osteocytes in the generation of human mineralized tissue.

1. Introduction

To date, most research aimed at understanding the underlying cellular processes involved in normal bone formation, remodeling or fracture repair rely on the use of either clinical samples or animal models, followed by morphological or biochemical analysis of harvested bone tissue. While these studies have yielded significant information on the effects of various local [1,2], systemic [3–5] and mechanical [6,7] stimuli in initiating or controlling bone remodeling, the use of such complex in vivo approaches hampers the investigation of the role played by individual cell types in this response. At its simplest level, the formation of mineralized bone tissue in vivo involves three major cell types, namely osteocytes, osteoblasts and osteoclasts, located in a structure within the intact bone known as the basic multicellular unit (BMU) [8]. Although the exact sequence of events remains unclear, there is consensus that both recruitment to and regulation of osteoclast and osteoblast activity at sites of bone remodeling involves a complex molecular cross-talk between all three major cell types. The overall response at the BMU in response to mechanical or hormonal stimuli in vivo results in bone remodeling, with osteocyte cells sensing changes in mechanical load, osteoclasts being responsible for bone resorption and osteoblasts being responsible for bone formation [8–12].

Previously, several monotype tissue culture models have been developed to simplify investigation of the underlying cellular mechanisms involved in bone remodeling. Osteoblast production of mineralized material has been studied in planar and three-dimensional tissue culture [13–15], while osteoclast resorption of mineralized material has been studied in culture utilizing pre-osteoclast cells grown on acellular bone material (i.e. dentin discs) in the presence of M-CSF and RANKL to induce osteoclastogenesis [16,17]. Naturally derived (i.e. acellular cadaver bone, collagen gels, coral, fibroin, etc.) or synthetic (i.e. polymers, ceramics, etc.) three-dimensional osteoconductive materials, several supplemented with recombinant bone morphogenic proteins (BMPs), have been used to investigate bone cell growth in vivo and in vitro [16,18–23]. However, the use of such foreign, exogenous

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materials to generate tissue culture analogs of bone tissue has hampered the investigation of the true sequence of cellular events which occur during de novo bone remodeling in vivo, especially as it relates to osteocyte involvement in "coupled" bone remodeling [24–26], or the confounding effects of exogenous osteoconductive scaffold material on the bone forming activity of these cells [23].

One general approach to studying the complex cellular interactions involved in tissue formation is the use of rotational culture to promote cell aggregation, cell–cell interaction and cellular differentiation similar to that observed within whole tissue [27]. Rotational culture has previously been used to investigate the differentiation and mineralization of both human and rodent osteoblast-like cells with [28–30] and without [14] the inclusion of a three-dimensional osteoconductive scaffolding material, while rotational culture has been used to study osteoclastogenesis and osteoclast resorptive activity in the presence of osteoblast cells grown on natural or synthetic polymer scaffolds [31,32]. It is well accepted that the formation of new bone material in vivo requires a balance between the activity of osteocytes, osteoblasts and osteoclasts located within a naturally occurring three-dimensional mineralized matrix to guide their growth and differentiation. However, to date a tissue culture analog which captures the complex three-dimensional multicellular interactions between the major cell types involved in bone formation/remodeling without the inclusion of a pre-existing exogenous osteoconductive scaffold material has yet to be developed.

We hypothesized that to generate a three-dimensional tissue culture analog of living bone tissue in the absence of any exogenous osteoconductive scaffolding material would require the presence of both osteoblast and osteoclast cells located in close proximity to each other as found during normal bone formation/remodeling in vivo. In addition, since osteoblasts/osteocytes cells far outnumber mature osteoclasts in normal remodeling bone in vivo, the relative proportions of osteoblasts and osteoclasts initially combined in culture should theoretically result in the number of differentiated osteoblasts far outnumbering that of differentiated osteoclasts in the final tissue analog. In order to test this hypothesis, we used solid body rotational culture to simultaneously co-culture primary human adult osteoblast and osteoclast precursor cells in the absence of any exogenous osteoconductive material to determine whether or not these culture conditions would promote the formation of a three-dimensional mineralized material. These culture conditions initially promoted direct cell–cell interaction and aggregation between the two original bone progenitor cell types resulting in the production of large (up to 4 mm in diameter) three-dimensional tissue aggregates in which the two original bone progenitor cell types underwent spatial organization. Sequential exposure of these tissue constructs in rotational culture to osteoclast differentiation factors and osteoblast mineralization agents induced the formation of a self-assembled mineralized extracellular matrix (ECM) spatially organized into two discrete zones: an external region in which reside both activated osteoblasts and osteoclasts, and a porous mineralized central region in which osteocytes are embedded. This unique in vitro analog of human bone allows investigation of not only the underlying cellular mechanisms involved in de novo bone formation/morphogenesis but also the effects of mechanical, hormonal and pharmaceutical stimuli on the unique three-dimensional interplay between bone forming cells and a native, self-assembled mineralized bone matrix.

2. Materials and methods

2.1. Human osteoblast (HOb) cell culture

Cryopreserved primary normal HOb cells were purchased from Lonza (Allendale, NJ) or PromoCell (Heidelberg, Germany) and expanded in planar culture using osteoblast basal medium (OBM) supplemented with 10% fetal calf serum (FCS), 5 μM sodium ascorbate and 100 μg ml^{-1} penicillin/100 IU ml^{-1} streptomycin (10%FCS.OBM; Lonza, Allendale, NJ). HOb cells were used for tissue construct production between passages 6 and 8. The ability of HOb cells to differentiate in our rotational cell culture system (RCCS; Synthecon Inc., Houston, TX) was initially confirmed by growing them on carrier beads in the RCCS for 7 days as previously described [33,34]. Bone-specific alkaline phosphatase (BAP) expression (a marker of osteoblast differentiation) was assessed by immunostaining using a mouse anti-human BAP monoclonal antibody (5 μg ml^{-1} IgG, Thermo Scientific, Rockford, IL) disclosed using a goat Alexa™ 488-conjugated goat anti-mouse polyclonal antibody (2 μg ml^{-1} IgG, Invitogen, Carlsbad, CA) after cell fixation using ice-cold Dulbecco's phosphate-buffered saline (D-PBS, pH 7.2) containing 1% (w/v) formaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 5 min. Immunostained cells were imaged using a Leica TCS scanning confocal microscope system (Leica Microscope Systems, Heidelberg, Germany).

2.2. Preparation of human osteoclast precursor (HOcP) cells

Normal HOcP cells (derived from human peripheral blood monocytes) were either purchased from Lonza (Allendale, NJ) as frozen cells or freshly isolated from human donor plasma buffy coats (Gulf Coast Blood Bank, Houston, TX) after Histopaque 1077 separation and negative selection by magnetic immune-bead separation using a human monocyte negative selection kit as per the manufacturer's instructions (Miltenyl Biotec, Auburn, CA). Freshly isolated human monocytes were frozen in freezing medium consisting of 10% dimethyl sulfoxide and 90%FCS.HOcP and stored under liquid nitrogen. HOcP cells were thawed and washed with fresh 10%FCS.OBM medium immediately prior to use. Alternatively, HOcP cells were pre-labeled with a fluorescent Cell Tracker™ dye (Invitogen, Carlsbad, CA) using a 1 μM solution dissolved in 10%FCS.OBM for 20 min at 37 °C as per the manufacturer's instructions. Cell Tracker™ dye labeling allowed subsequent identification and spatial localization of osteoclast cells within living tissue constructs using fluorescent scanning confocal microscopy. The ability of HOcP cells to differentiate into mature, multinucleated osteoclast cells expressing tartrate-resistant alkaline phosphotase (TRAP, aka ACP5) after Cell Tracker™ dye labeling was confirmed by growing labeled cells in planar culture in 10%FCS.OBM containing human recombinant M-CSF (50 ng ml^{-1}) and human recombinant RANKL (50 ng ml^{-1}) (ProSpec, Rehovot, Israel) for 7 days. Parallel cultures of HOcP cells were either histochemically stained for the presence of TRAP (aka ACP5) using a commercial staining kit (Sigma–Aldrich, St. Louis, MO) or fixed with ice-cold 1% (w/v) formaldehyde/D-PBS (pH 7.2) for 5 min prior to examination of Cell Tracker™ dye labeling. Cells were imaged using either an Olympus BX-60 light microscope with an attached digital camera (TRAP staining) or a Leica TCS scanning confocal microscope system (Cell Tracker™ dye labeling).

2.3. Generation of tissue constructs

Tissue constructs of various sizes were generated in the absence of any foreign, exogenous scaffolding material using disposable 10 ml high-aspect-ratio vessels (HARV) in an RCCS operated in a 5% CO_2 humidified tissue culture incubator maintained at 37 °C, being careful to remove all air bubbles from the HARV vessel prior to the initiation of rotation. All constructs were generated using cell mixtures suspended in 10%FCS.OBM containing human recombinant M-CSF (50 ng ml^{-1}) and human recombinant RANKL (50 ng ml^{-1}) inoculated into individual 10 ml HARVs. To date, tissue constructs have been generated using HOb and HOcP cells isolated from a to-