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Osteocytes up-regulate the terminal differentiation of pre-osteoblasts via gap junctions

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ABSTRACT

We examined cell-to-cell interaction between pre-osteoblasts and osteocytes using MC3T3-E1 and MLO-Y4, respectively. First, GFP expressing MC3T3-E1 (E1-GFP) cells were generated to isolate the cells from co-culture with MLO-Y4. No changes were observed in the expression of osteogenic transcription factors Runx2, Osterix, Dlx5 and Msx2, but expression of alkaline phosphatase (ALP) and bone sialoprotein (BSP) in E1-GFP co-cultured with MLO-Y4 was 300–400-fold greater than that in mono-cultured E1-GFP. In addition, mineralized nodule formation was drastically increased in co-cultured E1-GFP cells compared to mono-cultured cells. Patch clamp assay showed the presence of gap junctions between E1-GFP and MLO-Y4. Furthermore, when the gap junction inhibitor carbenoxolone (CBX) was added to the culture, increased expression of ALP and BSP in E1-GFP co-cultured with MLO-Y4 was suppressed. These results suggest that gap junction detected between pre-osteoblasts and osteocytes plays an important role on the terminal differentiation of pre-osteoblasts.

1. Introduction

Gap junctions, which mediate direct cell-to-cell-communication, play important roles in the maintenance of complex cellular functions. Disruption of these intercellular junctions leads to tissue dysfunction and a variety of physiological disorders [1]. Gap junctions are formed by the juxtaposition of two hemichannels composed of transmembrane proteins called connexins (Cx), and are localized to the plasma membrane where they can contact neighboring cells, thereby facilitating the rapid diffusion of small molecules and ions less than 1 kDa in a process known as gap junctional communication [2].

Bone is a dynamic tissue that undergoes constant remodeling in response to physical changes, including fluctuations in hormone levels, growth factors, and mechanical load [3]. Bone remodeling is constantly controlled by bone-resorbing osteoclasts and bone-forming osteoblasts, as well as osteocytes, which are embedded in the mineralized bone matrix [4]. In addition to endocrine, paracrine, and autocrine factors, direct cell-to-cell communication through gap junctions plays an important role in coordinating the activities of bone cells [5]. Recently, it was reported that osteocytes communicate with osteoblasts as well as other osteocytes through abundant gap junctions composed of Cx43. Gap junctions are permeable to molecules of up to 1.2 kDa, making them cable of propagating signals produced by ions, metabolites, and second messengers such as cAMP, cADP-ribose, and inositol derivatives [2]. Interestingly, disruption of the Cx43 gene in mice results in a delay in endochondral and intramembranous ossification. Calvarial osteoblasts isolated from Cx43 knockout mice demonstrate delayed expression of many osteoblast-specific markers, including osteocalcin (OCN), bone sialoprotein (BSP), and type I collagen [6]. Furthermore, manipulation of gap junctional communication by overexpression of Cx43 in the cultured osteoblastic cell lines alters basal expression of OCN, BSP, and alkaline phosphatase (ALP) in a reciprocal manner [7]. These findings strongly suggest that gap junctions are a fundamental part of the regulatory mechanism that controls the gene expression of osteoblast-specific markers during osteoblast differentiation and bone remodeling [2]. However, it has not been demonstrated whether osteocytes regulate osteogenic gene transcription and terminal differentiation of osteoblasts via gap junctions.

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Therefore, to understand the role of osteocytes as a micro-environmental factor in the terminal differentiation of pre-osteoblasts, we examined direct cell-to-cell interaction between MC3T3-E1 and MLO-Y4 cells in a unique co-culture system. The preosteoblast-like cell line MC3T3-E1 was derived from normal mouse bone tissue. MLO-Y4 cells are a line isolated from osteocalcin promoter-driven T-antigen transgenic mice and exhibit a stable osteocytic phenotype. When MLO-Y4 and MC3T3-E1 cells were co-cultured, the mRNA expression levels of ALP and BSP were strongly increased in MC3T3-E1 cells through gap junctions with MLO-Y4, and this was followed by enhanced mineralization in culture. Interestingly, increased ALP and BSP expression was not accompanied by an increase in the expression of osteogenic transcription factors, such as Runx2 and Osterix. These results suggest that cell-to-cell communication between pre-osteoblasts and osteocytes is sufficient for increased expression of ALP and BSP, and for subsequent terminal differentiation of pre-osteoblasts.

2. Materials and methods

2.1. Cells and reagents

All cells were maintained in growth medium composed of α-MEM (Wako, Osaka, Japan), 10% fetal bovine serum (FBS; Japan Bioserum Tokyo, Japan), and 1% penicillin–streptomycin (Wako, Osaka, Japan) at 37 °C in an atmosphere containing 5% CO_2. Carbenoxolone (CBX) was purchased from Sigma (St. Louis, MO).

2.2. Generation of GFP-expressing cells

MC3T3-E1 cells were seeded in a 6-well plate at a density of 1×10^5/well, and cultured for 18 h. Each well was then incubated with 2 ml of α-MEM containing 6.25 μl of Lipofectamine LTX (Invitrogen, Carlsbad, CA) with 2 μg of GFP-expression vector (pEGFP-N1, Takara Bio, Shiga, Japan) for 24 h. GFP-expressing cells were selected by culturing with G418 (2 mg/ml, Sigma, St. Louis, Mo), and their purity was confirmed by flow cytometry (BD FACS Aria; BD Biosciences, San Jose, CA).

2.3. Co-culture of E1-GFP and MLO-Y4 cells

An aliquot of 2×10^6 MLO-Y4 cells were gently mixed with 2×10^6 of E1-GFP cells in a 15 ml tube. The mixed cells were seeded into 10 cm dishes with growth medium and cultured for 1–72 h. For the ratio-dependent assay, cell numbers of each type cell were seeded for co-culture as shown in Supplemental Table 1. GFP-expressing cells were isolated using a FACS Aria (BD Biosciences). E1-GFP cells at 4×10^6 cells per well were used as a control. For separated culture experiments, E1-GFP cells were cultured at a density of 1×10^6 in a well covered by a filter chamber (1 μm pore size; Becton and Dickinson, Franklin Lakes, NJ) placed on 1×10^6 of MLO-Y4 cells for 24 h.

2.4. Whole-cell patch-clamp recording

Cultured cells were transferred to a recording chamber that was continuously perfused with a solution containing 126 mM NaCl, 3 mM KCl, 2 mM $MgSO_4$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 2.0 mM $CaCl_2$, and 10 mM D-glucose. Electrical signals were recorded by amplifiers (Axoclamp 700B, Axon Instruments, Foster City, CA), digitized (Digidata 1422A, Axon Instruments), observed on-line and stored on a computer hard disk using Clampex (pClamp 10, Axon Instruments). The composition of the pipette solution for recordings was as follows: 70 mM of potassium gluconate, 70 mM of KCl, 10 *N*-(2-hydroxyethyl) piperazine-*N*′-2-ethanesulfonic acid (HEPES), 15 mM of biocytin, 0.5 mM of EGTA, 2 mM of $MgCl_2$, 2 mM of magnesium adenosine triphosphate (ATP), and 0.3 mM of sodium guanosine triphosphate (GTP). Thin-wall borosilicate patch electrodes (2–5 MΩ) were pulled on a Flaming-Brown micropipette puller (P-97, Sutter Instruments, Novato, CA). Recordings were obtained at 30–31 °C. Membrane currents and potentials were low-pass filtered at 5–10 kHz and digitized at 20 kHz.

2.5. Analysis of mRNA expression

Total RNA was isolated using RNAiso Plus (Takara Bio) according to the manufacturer's instructions. First-strand cDNA was synthesized as previously reported [8]. cDNA was then diluted five-fold in sterile distilled water and 2 μl of cDNA was subjected to real-time RT-PCR using SYBR Premix Ex Taq™ II (Takara Bio) on a CFX96 Real-Time System (Bio-Rad Laboratories, Hercules, CA). The primer sets used are described in Supplemental Table 2. Each reaction was performed three times using cDNAs from different sample RNAs. mRNA expression levels were normalized to that of β-actin.

2.6. Noggin and CBX treatment

E1-GFP cells were co-cultured with MLO-Y4 cells in the presence or absence of recombinant mouse noggin (1 mg/ml) and cultured for 12 h. After incubation, the E1-GFP cells were isolated and subjected to real-time RT-PCR analysis. For CBX treatment, E1-GFP cells and MLO-Y4 cells were pre-cultured in growth medium in the presence or absence of CBX (100 μM) for 1 h, and then co-cultured for 12 h.

2.7. Cell counting assay

After co-culture, E1-GFP cells were isolated with a flow cytometer, seeded onto 100 mm culture dishes (1×10^4 cells/dish) and cultured for the indicated periods in the complete medium. The cells were then trypsinized and resuspended in the media, and the number of cells was determined using a microscope counting chamber (hemocytometer).

2.8. Cell cycle analysis

Cell cycle analysis was performed using Click-iT™ EdU Flow Cytometry Assay Kits (Invitrogen/Molecular Probes, Eugene, OR) according to the manufacturer's instructions. In brief, co-cultured cells were treated with 10 μM EdU (5-ethynyl-2′-deoxyuridine) for 1 h. EdU-incorporated cells were fixed with paraformaldehyde for 15 min and permeabilized for 30 min with a saponin-based buffer. The cells were treated for 30 min with the click-reaction mixture containing pacific blue azide, and resuspended in PBS buffer. GFP-positive cells were analyzed on a FACS Aria.

2.9. Alizarin Red S staining

After co-culture, E1-GFP cells were isolated with a FACS Aria and then cultured in 12-well plates with osteogenic medium for the indicated days. The osteogenic medium was composed of α-MEM supplemented with 10% FBS, 50 μg/ml L-ascorbate phosphate (Sigma), and 10 mM β-glycerophosphate (Sigma). Fresh osteogenic medium was added every 2 days. Thereafter, cultures were fixed in 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.3) for 30 min, washed with 0.1 M cacodylate buffer (pH 7.3), and stained for 5 min with a saturated solution of Alizarin Red S (pH 4.0). The wells were then washed with sterilized distilled water, dried, and examined under an EPSON GT-X800 scanner.

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