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Cancellation of the Calcification in Cultured Osteoblasts by CLEC-2

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Abstract: This study aims to investigate the effect of CLEC-2 on calcification in cultured mouse osteoblasts. In the RT-PCR and cell ELISA analysis, it was confirmed that osteoblasts express podoplanin, osteopontin, osteocalcin and sclerostin in culture, and that expressions of osteopontin and osteocalcin increased in calcification medium. The expression of podoplanin, osteopontin, osteocalcin and sclerostin did not change in osteoblasts with CLEC-2, indicating that CLEC-2 does not affect the expression of these bone proteins in osteoblasts. However, the amounts of calcified nodules and alkaline phosphatase activity were significantly suppressed in cultured osteoblasts by CLEC-2. The quantitative analysis showed that both the calcified nodule amount and alkaline phosphatase activity decreased with CLEC-2 while there was no influence in the cell viability with CLEC-2. Further, the expression of RUNX2 was observed in cytoplasm and in nucleus of cultured mouse osteoblasts while the expression decreased with CLEC-2. In Matrigel-based three-dimensional culture a significant cell process elongation of osteoblasts was observed and the elongation was strongly suppressed with CLEC-2. Considering these, CLEC-2 may have an ability to cancel the calcification of osteoblasts by blocking the maturation of osteoblast via interaction with CLEC-2 receptor podoplanin without any involvements of bone-associated protein production.

Key words: Podoplanin, CLEC-2

Introduction

Podoplanin, a mucin-type O-glycosylated type I transmembrane protein with highly negatively charged sialic acid contents, is a ligand to the transmembrane protein C-type lectin-like receptor CLEC-2 which is a hemITAM family member^{1,2)}. Podoplanin has functional residues including O-glycosylated Thr52 for binding to CLEC-2. Podoplanin binding with CLEC-2 results in association of cytoplasmic portions of podoplanin with CD44 on the podoplanin-expressing cell via ezrin-radixin-moesin (ERM) assembly³⁾. Intact platelets agglutinate around podoplanin-expressed cells via the recognition both the O-linked sugar and peptide in podoplanin. The CLEC-2 is expressed on platelets, megakaryocytes and mature dendritic cells while podoplanin is expressed on several cells such as fibroblastic reticular cells in lymph nodes, lymphatic endothelium, and tumor cells like oral cancer. Platelets, megakaryocyte fragments of 1–4 µm in diameter, are unable to synthesize proteins but retain the functional characteristics such as blood coagulation^{4,6)}. Binding of podoplanin to CLEC-2 causes the phosphorylation of the tyrosine residue of the YITL motif in the intracellular domains of CLEC-2 by the Src family kinase. Then, the tyrosine kinase Syk binds to the phospho-YITL motif and the Syk phosphorylates adapter SLP-76, eventually activating PLCγ2 and inducing platelet aggregation^{7,8)}. The aggregation of intact platelets around cancer cells, especially via CLEC-2 binding to podoplanin-expressing cells, promotes hematogenous metastasis¹⁻⁶⁾. The platelet aggregation at the venous an-

gle of the anterior cardinal vein during the embryonic period aids the separation of lymphatic vessels from the venous angle^{8,9)}. Migratory dendritic cells relax lymph nodes by binding of CLEC-2 to podoplanin in the lymph nodes¹⁰⁻¹³⁾. Fibroblastic reticular cells in lymph nodes produce collagen and contract the network. The cell contraction is dependent on the property of podoplanin of which causes ERM phosphorylation. The phospho-ERM-induced actomyosin rearrangements in fibroblastic reticular cells result in the cell contraction or cell process elongation. Upon inflammation, CLEC-2 on mature dendritic cells binds to podoplanin on fibroblastic reticular cells and potentially attenuate podoplanin-mediated contraction, resulting in lymph node expansion¹⁰⁻¹³⁾.

The earliest reported podoplanin gene for bone-related cells is OTS-8, a partial cDNA cloned from mouse osteoblast-like MC3T3-E1¹⁴⁾. The OTS-8 was cloned from the early response protein cDNA library of mouse osteoblast-like MC3T3-E1 cells treated with the tumor promoter 12-O-tetradecanoylphorbol-13-acetate, and Farr et al. reported that the 38 amino acid sequence epitope gp38 of mouse thymic epithelium recognized by a hamster monoclonal antibody derived from clone 8.1.1 closely resembles OTS-8¹⁵⁾. Further, podoplanin was established as an E11 antigen that is recognized by a monoclonal antibody for the rat osteoblastic osteosarcoma cell line ROS17/2.8 cells¹⁶⁻¹⁸⁾. Podoplanin is also produced from bone cells. Mature late osteoblasts and osteocytes produce podoplanin, and MC3T3-E1 and human osteoblast-like cells MG63 increase the expression of podoplanin in calcification medium¹⁷⁻²¹⁾. As mature osteoblasts and osteocytes express podoplanin at the plasma membrane of cell processes and bone extract contains a large amount of podoplanin, there may be soluble podoplanin which participates in the crystal sheath to promote the maturation of the mineralized cells

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Table 1. Sequence of primers

RefSeq NM_	mRNA	forward (5'-3') reverse (5'-3')	Tm (°C)	bp
7393	Actb	GTTCTACAAATGTGGCTGAGGA ATTGGTCTCAAGTCAGTGACAG	60.1 58.8	411
10329	Pdpn	CACCTCAGCAACCTCAGAC ACAGGGCAAGTTGGAAGC	54.5 54.2	192
7541	Bglap	GCAGACACCATGAGGACCATCTT GGGACTGAGGCTCCAAGGTAG	60.1 58.8	176
1204201	Spp1	CCATCTCAGAAGCAGAATCTCCTT GTCTCCATCGTCATCATCATCGT	58.0 58.0	150
24449	Sost	CTGCCTTCTGTGCTGTGGAG GGACACATCTTTGGCGTCATAGG	57.9 59.2	176

Gene symbols are described according to international notation.

nodule. Podoplanin has an increased expression in osteocyte dendrites, more than dentin matrix protein 1 (DMP-1) and sclerostin in the mature osteoblast/osteocyte²⁰⁻²². Mouse long bone osteocyte-like cells MLO-Y4 and IDG-SW3 express podoplanin more strongly than MC3T3-E1²¹⁻²⁴. Differentiation of cultured mouse calvarial osteoblasts and pre-osteoblastoid cell MLO-A5 into osteocytes in calcification medium containing β -glycerophosphate and ascorbic acid, and biomaterial-induced calcification are simultaneously accompanied by an increase in podoplanin²⁵⁻²⁸. Also, in the mature osteoblast MLO-A5 cell line, an increase in podoplanin coincides with dendrite formation, calcification, and RhoA activation²⁹. In the tooth germ, podoplanin is expressed on the enamel cord, cervical loop, internal and external enamel epithelium, and odontoblasts³⁰⁻³². Pdpn cKO mice show impairment in the cortical microarchitecture of tibia and in bone cells compared to fl/fl mice; lower cross-sectional thickness and lengths; decreased volume and lengths of dendrites. Thus, it is thought that podoplanin contributes to calcification via the osteocyte dendrite elongation³³. Since the cell shape and cytoskeleton arrangement are controlled by ERM-actomyosin assembly via podoplanin expression and by the separation via podoplanin-CLEC-2 binding^{3,10-13}, it is thought CLEC-2 may function as the counter-receptor for podoplanin to reduce calcification. This study aims to investigate the cancellation of calcification by CLEC-2 in cultured osteoblasts.

Materials and Methods

Cell culture

We purchased mouse bone marrow osteogenic stromal cells which (Cosmo Bio Co., Ltd, Tokyo, Japan) and induced osteoblasts with a mouse osteogenesis culture medium kit (Cosmo Bio) according to the supplier recommendations as also reported elsewhere³⁴. Osteoblasts were maintained in Minimum Essential Medium Eagle, Alpha Modification (α -MEM, Sigma-Aldrich Co. LLC., St. Louis, MO) with 10% fetal bovine serum (Biowest, Nuaille, France). For the test of the production of bone cell proteins and calcification, the cells were seeded on collagen-coated 24-well culture plates (Asahi Glass Co., Ltd., Shizuoka, Japan) in calcification medium of a mouse osteogenesis culture medium kit (Cosmo Bio) containing 100 nM dexamethasone, 50 μ g/ml, ascorbic acid, and 10mM β -glycerophosphate and further in the medium containing 0.1 and 1 μ g/ml the recombinant intact mouse CLEC2 (Catalog#9117-CL-050, R&D Systems Inc., Minneapolis, MN). The CLEC-2 binding ability to podoplanin was described in the manufacturer data sheet for the functional ELISA: the CLEC-2 binds with a typical ED50 of 0.1-0.5 μ g/ml on recombinant mouse podoplanin (Fc chimera, #3244-

PL, R&D Systems) coated at 1.0 μ g/ml (100 μ l/well) in a 96-well microtiter plate. Therefore, the dose of CLEC-2 was determined according to the ED50 of CLEC-2 with podoplanin and to the recent report performing CLEC-2 binding test⁶. In the study for measurement of RhoA activity, the direct activation of RhoA was evaluated using functional cell ELISA that living cells at 30% to 50% confluence on flat-bottomed plates were treated with 100 ng/ml CLEC-2.

Reverse transcription (RT)-PCR and real-time PCR

The RT-PCR and the real-time RT-PCR analysis for bone cell podoplanin, osteopontin, osteocalcin, and sclerostin mRNAs were performed on cultured mouse osteoblasts with CLEC-2. Total RNA extraction from the 80% confluent monolayer of osteoblasts (Cosmo Bio) cultured in α -MEM, in a mouse osteogenesis culture medium kit (Cosmo Bio) and in the medium containing 0.1 and 1 μ g/ml CLEC-2 (R&D Systems) in collagen-coated 24-well culture plates was performed with a QIAshredder column and RNeasy kit (Qiagen, Inc., Tokyo, Japan). Contaminating genomic DNA was removed using DNAfree (Ambion, Huntingdon, UK), and the RT was performed on 30 ng of total RNA, followed by 30 cycles of PCR for amplification using the Ex Taq hot start version (Takara Bio Inc., Otsu, Japan) with 50 pM of primer sets for mouse mRNA of β -actin, podoplanin, osteopontin, osteocalcin, and sclerostin, where the specificities had been confirmed by the manufacturer (Table 1) (Sigma-Aldrich Corp., Tokyo, Japan). The RT-PCR products were separated on 2% agarose gel (NuSieve; FMC, Rockland, ME, USA) and visualized by Syber Green (Takara). The correct size of the amplified PCR products was confirmed by gel electrophoresis and amplification of accurate targets was confirmed by a sequence analysis. To quantify the mRNA generation, cDNA samples were analyzed by real-time quantitative PCR. A total of 1 μ l of cDNA was amplified in a 25- μ l volume of PowerSYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in a Stratagene Mx3000P real-time PCR system (Agilent Technologies, Inc., Santa Clara, CA, USA), and the fluorescence was monitored at each cycle. Cycle parameters were 95°C for 15 min to activate Taq followed by 35 cycles of 95°C for 15 s, 58°C for 1 min, and 72°C for 1 min. For the real-time analysis, two standard curves were created from amplicons for both the β -actin and target genes in three serial 4-fold dilutions of cDNA. The β -actin or target gene cDNA levels in each sample was quantified against β -actin or the target gene standard curves by allowing the Mx3000P software to accurately determine each cDNA unit. Finally, target gene cDNA units in each sample were normalized to β -actin cDNA units. Relative target gene expression units were expressed as arbitrary units, calculated according to the following

Figure 1. Analysis for mRNAs of bone-associated proteins in mouse osteoblasts with CLEC-2. (A) RT-PCR. (B) real-time RT-PCR. Quantitative gene analysis was performed on mRNAs of control β -actin (β -actin), podoplanin (PDPN), osteopontin (OPN), osteocalcin (OCN), and sclerostin (SCN) in mouse osteoblasts cultured in α -MEM (α -MEM), in calcification medium (calcification), in calcification medium with 1 μ g/ml or 0.1 μ g/ml of CLEC-2. The expression amounts of PDPN and SCN mRNAs were at the same level in culture with α -MEM, calcification medium, and calcification medium with 1 μ g/ml and 0.1 μ g/ml CLEC-2. The expression amounts of OPN and OCN mRNAs were significantly larger in the culture with calcification medium than in α -MEM or in calcification medium with 1 μ g/ml and 0.1 μ g/ml CLEC-2. The expression amounts of PDPN, OPN, OCN and SCN mRNAs were at the same level in culture with calcification medium with 1 μ g/ml and 0.1 μ g/ml CLEC-2. The vertical axis is in arbitrary units. *Significantly different in ANOVA ($P < 0.01$).

formula: relative experimental gene expression units = experimental cDNA amounts of cells cultured in the calcification medium / control cDNA amounts of cells cultured in α -MEM.

Enzyme-linked immunosorbent assay (ELISA)

The cell ELISA analysis for podoplanin, osteopontin, osteocalcin, and sclerostin proteins was performed on cultured mouse osteoblasts with CLEC-2. The protein production amounts were investigated for the 80% confluent monolayer of osteoblasts (Cosmo Bio) cultured in α -MEM, in a mouse osteogenesis culture medium kit (Cosmo Bio) and in the medium containing 0.1 and 1 μ g/ml CLEC-2 (R&D Systems) in collagen-coated 24-well culture plates. The monolayer was incubated with primary antibodies (1 μ g/ml): hamster monoclonal anti-mouse podoplanin (BioLegend), rabbit anti-mouse osteopontin (Abcam), rabbit anti-mouse osteocalcin (Abcam), and rabbit anti-mouse sclerostin (Abcam). After the treatment with primary antibodies the culture was washed three times in PBS for 10 min and treated with peroxidase-conjugated second antibodies (0.1 μ g/ml) for 1 hr at 20°C, and then visualized by an ABTS peroxidase substrate system (SeraCare Life Sciences, Inc. [KPL]) in the plates at 37°C and absorbance changes at 405 nm were measured by a microplate reader. The culture treated with only the second antibody served as blanks. The protein production amounts in culture were expressed as the mean absorbance of peroxidase metabolizing substrate of six wells. Relative protein expression units were expressed in arbitrary units, calculated according to the following formula:

relative protein expression units = experimental protein amounts of cells cultured in the calcification medium / control protein amounts of cells cultured in the α -MEM.

Test for calcification, alkaline phosphatase activity, and CLEC-2 cytotoxicity

The 80% confluent monolayer of osteoblasts (Cosmo Bio) in collagen-coated 24-well culture plates (Asahi Glass) were cultured for 20 days in α -MEM, in the mouse osteogenesis culture kit (Cosmo Bio) and in the medium containing 0.1 and 1 μ g/ml CLEC-2 (R&D Systems). The culture was fixed by 10% formalin-PBS and stained by 40mM alizarin red S (pH 4.2) using a Calcified Nodule Staining kit (Cosmo Bio) for 30 min at room temperature. The stained culture was washed 3 times by distilled water to remove the staining dye and 5% formic acid was added to dissolve the calcified nodules stained by alizarin red. The calcification amounts in culture were determined by the absorbance at 450 nm of the yellow formic acid solution. Relative calcification amounts were expressed in arbitrary units, calculated according to the following formula: relative calcification units = the stained solution of calcified nodules of experimental culture / the solution of control culture with α -MEM. The culture was also stained by an alkaline phosphatase staining kit (Cosmo Bio) and alkaline phosphatase activities were further determined by the absorbance of catalyzed p-nitrophenyl phosphate (pNPP, Sigma-Aldrich Japan KK, Tokyo, Japan) at 405 nm after treatment with 1.0 mg/ml pNPP solution diluted in 20 ml of 2M Tris-buffered saline

(pH 9.1). Relative alkaline phosphatase activities were expressed in arbitrary units, calculated according to the following formula: relative alkaline phosphatase activity units = activity of experimental culture / activity of control culture with α -MEM.

The CLEC-2 cytotoxicity for osteoblasts (Cosmo Bio) was investigated. The 80% confluent monolayer of osteoblasts (Cosmo Bio) in collagen-coated 24-well culture plates (Asahi Glass) were cultured for 20 days in α -MEM, in the mouse osteogenesis culture kit (Cosmo Bio) and in the medium containing 0.1 and 1 μ g/ml CLEC-2 (R&D Systems). The cell viability was tested using water soluble tetrazolium salts WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium) of Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Tokyo, Japan). The nicotinamide adenine dinucleotide produced by intracellular dehydrogenase in mitochondria reduces WST-8 to orange formazan dye via 1-methoxy-5-methylphenazinium methylsulfate. Since the amount of formazan dye as a reduction product of WST-8 is proportional to the number of cells that are alive, that number was estimated with optical density measurements at the 450 nm wavelength by a microplate reader. Relative number of alive cells were expressed in arbitrary units, calculated according to the following formula: relative number of alive cells = absorbance of experimental culture / absorbance of control culture with α -MEM.

Immunostaining

The 80% confluent monolayer of osteoblasts (Cosmo Bio) were cultured on coverslips for 20 days in the mouse osteogenesis culture kit (Cosmo Bio) and in the medium containing 1 μ g/ml CLEC-2 (R&D Systems). The cells were fixed in 100% methanol for 30 sec at -20°C , treated with 0.1% goat serum for 30 min at 20°C , and then reacted with 1 μ g/ml of rabbit anti-mouse Runx2-related transcription factor (RUNX2, Abcam) for 8 hrs at 4°C with PBS containing 0.1% goat serum. After the treatment with primary antibodies the cells were washed three times in PBS for 10 min and reacted for 0.5 hr at 20°C with 0.1 μ g/ml of second antibodies: Alexa Fluor 568-conjugated goat anti-hamster or goat anti-rabbit IgGs (Probes Invitrogen Com., Eugene, OR). The immunostained sections were mounted in 50% polyvinylpyrrolidone solution and examined by microscope digital camera systems with CFI Plan Apo Lambda lens series and DS-Ri2/Qi2 (Nikon Corp., Tokyo, Japan). The RUNX2-stained area was measured on five different field-of-view images of immunostained culture using ImageJ (National Institutes of Health, Bethesda, MD). The number of RUNX2-positive cells was estimated by the ratio of the immunostained area (%): positive area / scanned area. All experiments were repeated five times.

Test for the length of cell processes

The 80% confluent monolayer of osteoblasts (Cosmo Bio) was cultured in Matrigel (BD Biosciences, San Jose, CA)-based three-dimensional culture in 24-well culture plates (Asahi Glass) for 20 days in the mouse osteogenesis culture kit (Cosmo Bio) and in the medium containing 1 μ g/ml CLEC-2 (R&D Systems). The length of cell processes was examined by a phase-contrast microscope digital camera system with CFI Plan Apo Lambda lens series and DS-Ri2 (Nikon Corp., Tokyo, Japan).

Statistics

All experiments were repeated at least five times and the data was expressed as mean + SD. Statistically significant differences ($P < 0.01$) were determined by one-way ANOVA and the unpaired two-tailed Student's *t* test with STATVIEW 4.51 software (Abacus concepts, Calaba-

Figure 2. Analysis for the produced amounts of bone-associated proteins in mouse osteoblasts with CLEC-2. Protein amounts were quantified by Cell ELISA on podoplanin (PDPN), osteopontin (OPN), osteocalcin (OCN), and sclerostin (SCN) in mouse osteoblasts cultured in α -MEM (α -MEM), in calcification medium (calcification), in calcification medium with 1 μ g/ml or 0.1 μ g/ml of CLEC-2. The produced amounts of PDPN and SCN protein were at the same level in culture with α -MEM, calcification medium, and calcification medium with 1 μ g/ml and 0.1 μ g/ml CLEC-2. The produced amounts of OPN and OCN protein were significantly larger in the culture with calcification medium than in α -MEM or in calcification medium with 1 μ g/ml and 0.1 μ g/ml CLEC-2. The produced amounts of podoplanin, osteopontin, osteocalcin and sclerostin protein in cultured osteoblasts with calcification medium were at the same level with 1 μ g/ml and 0.1 μ g/ml CLEC-2. The vertical axis is in arbitrary units. *Significantly different in ANOVA ($P < 0.01$).

sas, CA, USA).

Results

The gene expression of bone-associated proteins in mouse osteoblasts with CLEC-2

The effect of CLEC-2 on the gene expression of bone-associated proteins in mouse osteoblasts were analyzed by RT-PCR (Fig. 1). The gene expression of podoplanin and sclerostin was at the same level in culture with α -MEM, calcification medium, and calcification medium with 1 μ g/ml and 0.1 μ g/ml CLEC-2. The gene expression of osteopontin and osteocalcin was significantly larger in the culture with calcification medium than in α -MEM or in calcification medium with 1 μ g/ml and 0.1 μ g/ml CLEC-2. The gene expression of podoplanin, osteopontin, osteocalcin and sclerostin was at the same level in culture with calcification medium with 1 μ g/ml and 0.1 μ g/ml CLEC-2.

The protein production of bone-associated proteins in mouse osteoblasts with CLEC-2

The effect of CLEC-2 on the production of bone-associated proteins in mouse osteoblasts were analyzed by cell ELISA analysis (Fig. 2). The

A

B

Figure 3. Calcification, alkaline phosphatase (ALP) activity, and cell viability of mouse osteoblasts with CLEC-2. (A) Alizarin red staining and ALP staining. Calcification was estimated by alizarin red staining in mouse osteoblasts cultured in α -MEM (α -MEM), in calcification medium (calcification), in calcification medium with 1 μ g/ml or 0.1 μ g/ml of CLEC-2. The amounts of calcified nodules stained by alizarin red were significantly larger in calcification medium than in α -MEM while they were significantly smaller in calcification medium with 1 μ g/ml and 0.1 μ g/ml CLEC-2 than in calcification medium without CLEC-2. ALP activity of cells cultured with CLEC-2 was estimated by ALP staining. The ALP-positive cells were significantly larger in calcification medium than in α -MEM while they were significantly smaller in calcification medium with 1 μ g/ml and 0.1 μ g/ml CLEC-2 than in the medium without CLEC-2. (B) Quantitative analysis for calcification, ALP activity and cell viability. All analysis was performed on mouse osteoblasts cultured in α -MEM (α -MEM), in calcification medium (calcification), in calcification medium with 1 μ g/ml or 0.1 μ g/ml of CLEC-2. Calcification amounts were quantified by the absorbance at 450nm of the formic acid solution of alizarin red-stained calcified nodules. Relative dissolved calcification amounts were expressed in arbitrary units, calculated according to the following formula: relative calcification units = the solution of experimental culture / the solution of control culture with α -MEM. The amounts of dissolved calcified nodules stained by alizarin red were significantly larger in calcification medium than in α -MEM while they were significantly smaller in calcification medium with 1 μ g/ml and 0.1 μ g/ml CLEC-2 than in calcification medium without CLEC-2. The amounts of dissolved calcified nodules were also significantly smaller in calcification medium with 1 μ g/ml CLEC-2 than in the medium with 0.1 μ g/ml CLEC-2. *Significantly different in ANOVA ($P < 0.01$). **Significantly different from culture in α -MEM, and different from each other in student t test ($P < 0.01$). ALP activity was quantified by the absorbance for hydrolysis of pNPP catalyzed by cellular intrinsic ALP at 405 nm. Relative ALP activities were expressed in arbitrary units, calculated according to the following formula: relative ALP activity units = activity of experimental culture / activity of control culture with α -MEM. The amounts of catalyzed pNPP were significantly larger in calcification medium than in α -MEM while they were significantly smaller in calcification medium with 1 μ g/ml and 0.1 μ g/ml CLEC-2 than in the medium without CLEC-2. The amounts of catalyzed pNPP were also significantly smaller in calcification medium with 1 μ g/ml CLEC-2 than in the medium with 0.1 μ g/ml CLEC-2. *Significantly different in ANOVA ($P < 0.01$). **Significantly different from culture in α -MEM, and different from each other in student t test ($P < 0.01$). The number of living cells (cell viability) was estimated by the amount of formazan dye as a reduction product of WST-8 with optical density measurements at the 450 nm wavelength. Relative numbers of alive cells were expressed in arbitrary units, calculated according to the following formula: relative number of alive cells = absorbance of experimental culture / absorbance of control culture with α -MEM. There were no statistically significant differences in the numbers of alive osteoblasts cultured in α -MEM, in the mouse osteogenesis culture and in the medium containing 0.1 and 1 μ g/ml CLEC-2 as determined by the ANOVA ($P < 0.01$), indicating that CLEC-2 did not affect the cell viability.

protein production of podoplanin and sclerostin protein was at the same level in culture with α -MEM, calcification medium, and calcification medium with 1 μ g/ml and 0.1 μ g/ml CLEC-2. The protein production of osteopontin and osteocalcin protein was significantly larger in the culture with calcification medium than in α -MEM or in calcification medium with 1 μ g/ml and 0.1 μ g/ml CLEC-2. The protein production of podoplanin, osteopontin, osteocalcin and sclerostin protein in cultured osteoblasts with calcification medium was at the same level with 1 μ g/ml and 0.1 μ g/ml CLEC-2.

Calcification, alkaline phosphatase activity and cell viability of mouse osteoblasts with CLEC-2

The effect of CLEC-2 on the calcification, alkaline phosphatase activity and cell viability of mouse osteoblasts were tested by the alizarin red staining, hydrolysis of pNPP catalyzed by alkaline phosphatase, and the reduction product of WST-8 (Fig. 3). In alizarin red staining the amounts of calcified nodules stained by alizarin red were significantly larger in calcification medium than in α -MEM while they were significantly smaller in calcification medium with 1 μ g/ml and 0.1 μ g/ml CLEC-2 than in calcification medium without CLEC-2. In alkaline

Figure 4. Nuclear localization of RUNX2 of cultured mouse osteoblasts. (A) Immunostaining. The mouse cultured osteoblasts were immunostained with anti-RUNX2 in cytoplasm and nuclei with calcification medium (without CLEC-2) while rarely stained in calcification medium with 1 μ g/ml CLEC-2 (with CLEC-2). Bar: 100 μ m. (B) Quantitative analysis of cells immunostained by anti-RUNX2. The Runx2-stained area was measured on five different field-of-view images of immunostained culture using ImageJ. The number of RUNX2-positive cells was estimated by the ratio of the immunostained area (%): positive area / scanned area. The number of RUNX2-positive cells was significantly smaller in calcification medium with 1 μ g/ml CLEC-2 (with CLEC-2) than in the medium without CLEC-2 (without CLEC-2). *Significantly different by unpaired student t test ($P < 0.01$).

Figure 5. Cell process elongation of cultured mouse osteoblasts in three-dimensional culture. (A) Phase-contrast microscopy. The osteocyte cell process elongation of osteocyte-like cells was observed in Matrigel-based three-dimensional culture with calcification medium (without CLEC-2) while the cell process elongation was suppressed in culture with 1 μ g/ml CLEC-2 (with CLEC-2). Bar: 100 μ m. (B) Quantitative analysis for the length of cell processes. The length of cell processes in three-dimensional culture was shorter in calcification medium with 1 μ g/ml CLEC-2 (with CLEC-2) than in the medium without CLEC-2 (without CLEC-2). *Significantly different by unpaired student t test ($P < 0.01$).

phosphatase staining the alkaline phosphatase-positive cells were significantly larger in calcification medium than in α -MEM while they were significantly smaller in calcification medium with 1 μ g/ml and 0.1 μ g/ml CLEC-2 than in the medium without CLEC-2. In the quantitative analysis for calcification the amounts of calcified nodules dissolved in the formic acid solution were significantly larger in calcification medium than in α -MEM while they were significantly smaller in calcification

medium with 1 μ g/ml and 0.1 μ g/ml CLEC-2 than in calcification medium without CLEC-2. The amounts of calcified nodules were also significantly smaller in calcification medium with 1 μ g/ml CLEC-2 than in the medium with 0.1 μ g/ml CLEC-2. In the quantitative analysis for alkaline phosphatase activity the amounts of catalyzed pNPP by cellular intrinsic alkaline phosphatase were significantly larger in calcification medium than in α -MEM while they were significantly smaller in calcifi-

cation medium with 1 µg/ml and 0.1 µg/ml CLEC-2 than in the medium without CLEC-2. The amounts of catalyzed pNPP were also significantly smaller in calcification medium with 1 µg/ml CLEC-2 than in the medium with 0.1 µg/ml CLEC-2. In the quantitative analysis for the CLEC-2 cytotoxicity on osteoblasts was also investigated. The number of living cells cultured with CLEC-2 was estimated by the amount of formazan dye as a reduction product of WST-8. There were no statistically significant differences in the numbers of alive osteoblasts cultured in α -MEM in the mouse osteogenesis culture and in the medium containing 0.1 and 1 µg/ml CLEC-2, indicating that CLEC-2 did not affect the cell viability in the experimental procedures.

Nuclear localization of RUNX2 of cultured mouse osteoblasts

The effect of CLEC-2 on the localization of RUNX2 in mouse osteoblasts was tested by the immunostaining with anti-RUNX2 (Fig. 4). The mouse cultured osteoblasts were immunostained with anti-RUNX2 in cytoplasm and nuclei with calcification medium while rarely stained in calcification medium with 1 µg/ml CLEC-2 (Fig. 4). The number of RUNX2-positive cells was significantly smaller in calcification medium with 1 µg/ml CLEC-2 than in the medium without CLEC-2.

Cell process elongation of cultured mouse osteoblasts

The effect of CLEC-2 on the cell process elongation of mouse osteoblasts was tested in Matrigel-based three-dimensional culture (Fig. 5). The cell process elongation of osteoblasts was observed in three-dimensional culture with calcification medium while the cell process elongation was suppressed in culture with 1 µg/ml CLEC-2. The length of cell process in three-dimensional culture was shorter in calcification medium with 1 µg/ml CLEC-2 than in the medium without CLEC-2.

Discussion

Effect of CLEC-2 on the production of bone-associated proteins in cultured osteoblasts

Mature osteoblasts secrete matrix vesicles into the bone matrix to induce calcification. Alkaline phosphatase in the matrix vesicles hydrolyzes pyrophosphate to form phosphate and takes up calcium to form calcium phosphate crystals. Apatite ribbons on the calcium phosphate crystal core forms the mineralized nodules and are covered by a crystal sheath composed of non-collagen protein like osteocalcin, osteopontin, and others³⁵. Osteoblasts produce alkaline phosphatase and osteopontin in the middle stage of maturation, and osteocalcin and podoplanin in late maturation³⁶⁻⁴⁰. Sclerostin is an antagonist for *Wnt* signaling, which is a protein encoded by the *Sost* gene primarily expressed. *Sost*/sclerostin is a typical protein for mature osteoblasts and the expression increases with maturing. Sclerostin is rarely expressed in osteoid osteocytes recently embedded in bone or in osteocytes close to bone forming surfaces, however, there is a high level of sclerostin in osteocytes surrounded by mineralized bone which is distant from active bone surfaces^{41,42}. This study showed that cultured cells expressed podoplanin, osteopontin, osteocalcin and sclerostin. The gene and protein expressions of osteopontin and osteocalcin increased in calcification medium, and the expressions of podoplanin and sclerostin were at the same level in α -MEM and in calcification medium, indicating that there were cells matured from early stage to late stage in culture (Figs. 1 and 2). This study also showed that expressions of podoplanin, osteopontin, osteocalcin and sclerostin did not change in osteoblasts with 1 µg/ml and 0.1 µg/ml CLEC-2, indicating that CLEC-2 does not affect the expression of these bone cell-associated proteins in osteoblasts (Figs. 1 and 2).

Effect of CLEC-2 on the calcification and maturation in cultured osteoblasts

Our previous study showed that the calcification of osteoblasts decreased by culturing with anti-podoplanin³⁴. Podoplanin plays an important role in cell process elongation and contraction by the actin cytoskeleton rearrangement dependent on the binding activity of the cytoplasmic portion of podoplanin with the cytoplasmic linker protein ezrin via RhoA family signaling. The expression of podoplanin in the cell membrane promotes binding of podoplanin to F-actin by phosphorylated ezrin-radixin-moesin assembly, resulting the formation of a cell membrane-actin structure and cell process extension, whereas CLEC-2 binding to podoplanin induces relaxation of cytoskeleton tension through dissociation of podoplanin-ezrin assembly¹³. In the present study the amounts of calcified nodules and alkaline phosphatase activity were suppressed in cultured osteoblasts by CLEC-2 (Fig. 3). The quantitative analysis also showed that both the calcified nodule amount and alkaline phosphatase activity decreased with CLEC-2, and that there was no influence in the cell viability with CLEC-2. Since CLEC-2 is well known as only a receptor of podoplanin as described above, these suggests that the calcification of osteoblasts was canceled in vitro by CLEC-2. The expression of RUNT-related transcription factor RUNX2 was observed in cytoplasm and in nucleus of cultured mouse osteoblasts while the expression decreased with CLEC-2 (Fig. 4). Further in Matrigel-based three-dimensional culture, a significant cell process elongation of osteoblasts was observed and the elongation was strongly suppressed with CLEC-2 (Fig. 5). The RUNX2 induces the osteoblast development from mesenchymal cells⁴³. The RUNX2 is a factor inducing differentiation of mesenchymal stem cells into osteoblasts and the *Runx2* deficient mice do not show bone formation. It has already been demonstrated that the podoplanin cKO suppressed bone cell process elongation resulting in the reduction of the ability to calcify. Therefore, it is thought that the suppression of cultured osteoblast process elongation caused a decrease in the calcification ability such as the reduction of alkaline phosphatase^{29,33}. Considering these, CLEC-2 may act as a cancellation factor for the calcification with blocking the osteoblast maturation via CLEC-2 receptor podoplanin.

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Conflicts of Interest

The authors state that there are no potential conflicts of interest.

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