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Review

Morphology, Function, and Differentiation of Bone Cells

Hiroaki Nakamura

Department of Oral Histology, Matsumoto Dental University. Shiojiri, Japan
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Abstract: Bone plays a pivotal role in storing calcium and phosphate in vertebrates. This tissue is maintained by the balance of bone formation and bone resorption. Osteoblast-lineage cells, consisting of osteoblasts, osteocytes and bone lining cells, are engaged in bone formation. Bone resorption is mediated by osteoclasts. Recent research revealed that receptor activator of NF-κB (RANK)-RANK ligand (RANKL) mechanism is essential for the differentiating and activating osteoclasts. Osteoblast-lineage cells regulate bone resorption *via* the expression of RANKL and osteoprotegerin (OPG), a decoy receptor for RANKL. Additionally, osteoblast-lineage cells participate in degradation of bone matrix by secreting MMP-13. Thus, bone remodeling is achieved by the harmonized orchestration of osteoblast-lineage cells and osteoclast-lineage cells.

Key words: Bone, Cell-cell interaction, Osteoblasts, Osteoclasts

Introduction

Bone is not inert tissue but dynamically metabolized connective tissue throughout life [1, 2]. Old bone matrices are always replaced by newly formed matrices. This continual process, named bone remodeling, is important for maintaining bone volume and strength. Bone volume is maintained by the balance of bone resorption and bone formation. Bone cells consist of osteoblast-lineage cells [3, 4] and osteoclast-lineage cells [5]. Their differentiation and function are regulated by osteotropic hormones and cytokines. Recent research has revealed that osteoblast-lineage cells are not only involved in bone formation but also in bone resorption via supporting differentiation and activation of osteoclasts [6]. Hence, we need to re-consider the functional and morphological varieties of osteoblast-lineage cells. This review describes morphological characteristics of osteoblast-lineage and osteoclast-lineage cells and also discusses their function and differentiation.

Morphology and function of osteoblasts

Osteoblasts are engaged in bone formation. They are generally round in shape and line on the bone surfaces (Fig.1A). Ultrastructural property of osteoblasts shows typical secretory characteristics, possessing well-developed rough endoplasmic reticulum with dilated cisterna [1, 2]. A large Golgi complex comprises multiple Golgi stacks, vesicles and vacuoles containing fibrillar structures which are considered to represent pro-collagen and proteoglycans (Fig. 1A). Newly formed bone matrix is not calcified immediately. Therefore, uncalcified matrix, named osteoid, exists under the regulation of active bone-forming osteoblasts. Quantity of osteoid is closely related with bone-forming activity of osteoblasts. Much osteoid are seen under actively bone-forming osteoblasts.

After active bone formation, some osteoblasts become osteocytes buried in bone matrix. Others exist on quiescent bone surfaces and are called as bone lining cells. Bone lining cells show flattened shape and contain a few cell organelles. These morphological characteristics indicate that bone lining cells are hardly engaged in bone formation. In fact, little osteoid is seen under the bone lining cells.

Osteocytes are considered to be the terminal differentiation stage of osteoblasts. They are embedded in osteocytic lacunae and are most abundant cells in bone tissue. Osteocytic lacunae are connected by canaliculi containing their cytoplasmic processes. These canaliculi serve as pathway to supply nutrients and oxygen from blood capillary to osteocytes. Osteocytes possess extremely large surface area because of numerous cytoplasmic processes. Additionally, these processes contain well-developed bundles of actin filaments receivable of mechanical stress. It is conceivable that osteocytes are involved in bone metabolism by receiving and transducing mechanical stress. In fact, recent research revealed that osteocytes express stretch activated channel [7] and shear-stress-responsive element [8]. However, mechanism of signal transduction and genes regulated by mechanical stress are not clarified yet.

Bone consists of 70% inorganic component, 20% organic component, and 10% water. Approximately 90% of organic content is type I collagen. Osteoblasts are responsible for the production of collagen and non-collagenous proteins including osteocalcin, bone sialoprotein, osteopontin, and osteonectin [3, 4]. They also

Correspondence toDr. Hiroaki Nakamura, Department of Oral Histology, Matsumoto Dental University, 1780 Gobara Hirooka, Shiojiri, Nagano 399-0781, Japan. Tel.:+81-263-51-2042; Fax:+81-263-53-3456.E-mail address: nakam@po.mdu.ac.jp.

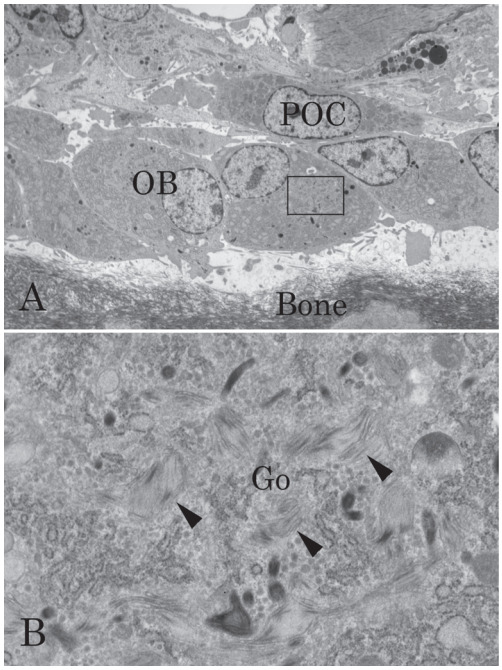

Fig. 1 Electron micrographs of osteoblasts.
A: Round osteoblasts (OB) are seen on bone matrix (Bone). POC; preosteoclast. B: Electron micrograph, at a higher magnification, of square in A. Golgi apparatus (Go) of osteoblasts consists of cistern, vesicles, and vacuoles containing fibrilar structures (arrowhead).

synthesize and secrete proteoglycans such as decorin and biglycan. Since these glycoproteins and proteoglycans could bind calcium ion, they are considered to be involved in two functions: storing calcium ion for calcification and regulating growth of hydroxyapatite by preventing excess calcification. Osteoblasts also produce cytokines including insulin-like growth factor I, II, transforming growth factor β (TGF-β), and bone morphogenetic proteins (BMPs) [3]. These growth factors are stored in calcified bone matrix and play an important role in differentiation and function of osteoblasts. Thus, bone matrix acts as a storage site of growth factors in addition to calcium and phosphates.

Osteoblasts demonstrate intense alkaline phosphatase activity on their plasma membrane. This histochemical feature has been used for a marker of osteoblast-lineage cells. Recent research of tissue non-specific alkaline phosphatase (TNAP)-deficient mice revealed that TNAP acts as pyrophosphatase hydrolyzing pyrophosphate, inhibitor of calcification, and increases the concentration of inorganic phosphates required for calcification [9]. Although ALPase activity is intense in the basolateral plasma membrane of osteoblasts, their membrane towards osteoid and the plasma membrane of osteocytes hardly show ALPase activity. This histochemical evidence indicates that the distribution of ALPase does not always correlate with calcification sites. Moreover, calcification is not completely disturbed in TNAP-deficient mice. Thus, precise function of ALPase in osteoblasts remains controversial.

Cell-cell interaction among osteoblast-lineage cells is important for their differentiation and function. Arana-Chavez et al.[10] reported three types of cell adhesion in osteoblasts at early developmental stage of calvaria by electron microscopy: focal tight junctions, adherens junctions, and gap junctions. Tight junctions are thought to be involved in maintaining cell polarity and preventing macromolecules to enter the intercellular spaces. Continuous tight junctions, also called a *zunula occludens*, are widely seen in epithelial cells. However, there is no continuous tight junction in osteoblasts. This evidence suggests that tight junctions in osteoblasts may not play a role in segregation of bone matrix from extracellular fluid. Gap junctions in osteoblast-lineage cells are mainly composed of connexin 43. Osteoblasts, bone lining cells, and osteocytes are connected by their cell processes through gap junctions. These junctions are involved in transport of ions and micromolecules among osteoblast-lineage cells. Thus, gap junctions are engaged in synchronized function of osteoblast-lineage cells to respond to various physiological signals. Adherens junctions are composed by cadherins. Major cadherins expressed in osteoblasts are N-cadherin and cadherin-11 (Ob-cadherin). In addition to mechanical function, adherens junctions are thought to be involved in signal transduction via cell-cell interaction because they are associated with β-catenin as well as tyrosin-kinases [11]. Moreover, bone formation rate and bone volume are decreased in cadherin-11-deficient mice [12]. These facts suggest that cell-cell adhesion via cadherins could contribute to regulation of differentiation, function and survival of osteoblasts.

Cell-matrix interaction between osteoblasts and bone matrix proteins, including type I collagen, non-collagenous proteins, and fibronectin, is also important for differentiation and function of osteoblasts. The interaction between β_1 integrins, $\alpha_1\beta_1$ and $\alpha_2\beta_1$, and type I collagen plays a key role in differentiation and function of osteoblasts via activation of mitogen-activated protein kinase (MAPK) signaling pathway [13]. Moreover, numerous cytoplasmic processes of osteoblasts extend into osteoid. The orientation of collagen fibers in lamellar bone alternates from layer to layer. This lamellar structure might be determined by cell-matrix adhesion between osteoblasts' process and collagen.

Recent works have revealed that osteoblast-lineage cells are involved in differentiation and activation of osteoclasts as well as bone formation. Macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor (NF)-kB ligand (RANKL) essential for osteoclastogenesis are expressed in osteoblast-lineage cells [6]. In addition, osteoprotegerin (OPG), decoy receptor for RANKL, is secreted by osteoblasts. Bone resorption by osteoclasts might be regulated by the balance of RANKL and OPG expressed in osteoblast-lineage cells.

On the other hand, osteoblasts and osteocytes secrete matrix metalloproteinase (MMP)-13, indicating that osteoblast-lineage cells are engaged in degradation of collagen[14]. Sakamoto and Sakamoto[15] reported that osteoblasts and osteocytes participate

in bone resorption by secretion of collagenase. Indeed, osteoclasts form deeper resorption lacunae in living bone than those in devitalized bone. Fewer collagen fibrils in living bone suggest collagenase secretion by osteoblast-lineage cells[16]. Additionally, in situ hybridization studies have shown that mRNA of MMP-13 is expressed in osteoblast-lineage cells[17]. Immunolocalization of MMP-13 protein is detected on the bone surface of Howship's lacunae and osteocytes adjacent to osteoclasts, but hardly seen in actively bone-forming osteoblasts and osteoclasts[14]. Immunogold labeling for MMP-13 is detected in Golgi apparatus of osteocytes under osteoclasts and bone canaliculi, indicating that MMP-13 is secreted by osteocytes and translocated into Howship's lacunae through the lacunae-canaliculi channel. Taken together, collagen fibrils may be fragmented by MMP-13 produced by osteoblast-lineage cells, and further degraded to low-molecules by MMP-9 secreted by osteoclasts[18]. These findings suggest that osteoblast-lineage cells participate in degradation of collagen during bone resorption in concert with osteoclasts. Furthermore, parathyroid hormone (PTH) regulates the MMP-13 promoter in osteoblast-lineage cells via activator protein (AP)-1 and Runx 2 binding sites [19, 20]. This mechanism might be partly involved in enhancement of bone resorption by PTH.

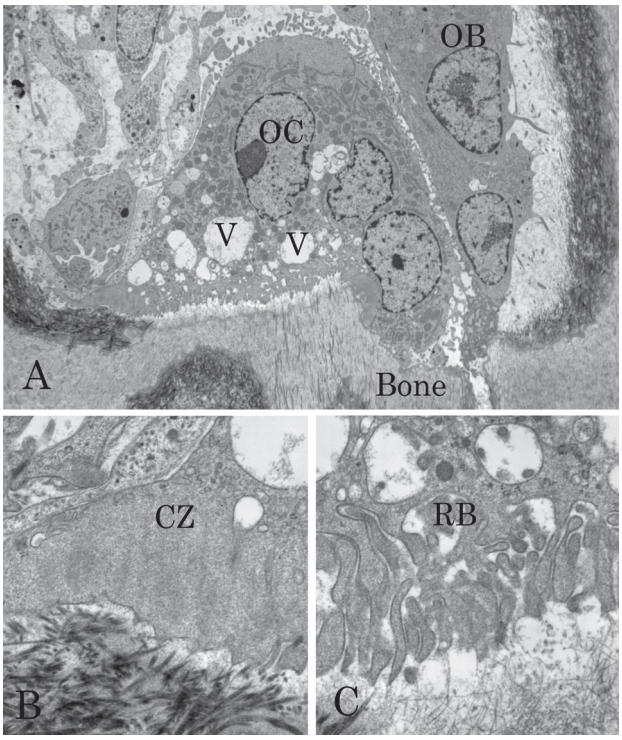

Fig. 2 Electron micrographs of an osteoclast.
A: A multinucleated osteoclast attaches bone surface (Bone). Numerous mitochondria and vacuoles (V) are seen in its cytoplasm.
B: Clear zone (CZ) contains networks of actin filaments.
C: Ruffled border (RB) shows finger-like cytoplasmic processes.

Differentiation of osteoblasts

Osteoblasts originate from mesenchymal stem cells (MSCs). MSCs could differentiate into chondrocytes, osteoblasts, myoblasts, and adipocytes[4]. Their differentiation is regulated by specific transcription factors. Sox 5, 6 and 9 regulate chondrocytic differentiation. Differentiation of adipocytes and myoblasts is determined by PPARγ and Myo D, respectively. In case of osteoblast-lineage cells, Runx 2/Cbfa[21, 22] and Osterix/Sp7[23] are essential regulators. Runx 2-deficient mice can not develop bone tissue. Cleidocranial dysostosis showing abnormality in membranous ossification is caused by defect in Runx 2 gene. Although Runx 2 is found as transcriptional factor binding to osteocalcin promoter, it also contributes to the gene expression of osteopontin, bone sialoprotein, dentin sialoprotein, and TGFβ receptor I.

Osteoblast-lineage cells show stepwise expressions of their specific markers including matrix proteins and ALPase in the process of their differentiation. Differentiation and function of osteoblast-lineage cells are regulated by hormones, including 1, 25$(OH)_2D_3$, PTH, and estrogen, and cytokines such as BMPs. BMPs, BMP-2, BMP-4, and BMP-7, induce osteogenesis in vivo and in vitro[24]. BMPs were discovered as osteo-inductive factors in decalcified bone and dentin by Urist et al.[25] BMPs prevent multipotential muscle satellite cells to differentiate into myoblasts and adipocytes, and, in turn, promote chondrocytic and osteoblastic differentiation. These phenomena are closely related with the somite derived from mesoderm. Somite contains multipotential cells to undergo myogenic, osteogenic, and adipogenic differentiation. BMPs conduct endochondral ossification by inducing mesenchymal cells to differentiate into osteogenic cells.

In signal transduction pathway of BMPs, their specific receptor complex leads to phosphorylation of Smads (R-Smads) that form heterodimer with Smad4 and regulate gene expression. BMP activity is also regulated by inhibitory Smads (I-Smads) and antagonists including noggin, chordin and sclerostin[4]. Although BMPs promote differentiation of osteoblasts by preventing MyoD expression[26] and inducing Runx 2 expression, precise transcriptional mechanism has not been clarified yet.

Recent reports suggest that the canonical Wnt/β-catenin pathway is involved in early development by promoting osteoprogenitor differentiation[27]. Furthermore, cell-cell and cell-matrix interaction participate in functional and morphological changes of osteoblast-lineage cells. Numerous factors might be involved in the differentiation of osteoblasts.

Function and morphology of osteoclasts

Osteoclasts are multinucleated giant cells responsible for bone resorption. Active osteoclasts come in contact with calcified bone surface and exist within Howship's lacunae which are eroded by their own resorptive activity[1]. Osteoclasts are generally distinguished from other bone cells by their large size and multiple nuclei (Fig 2A). Their ultrastructures show numerous

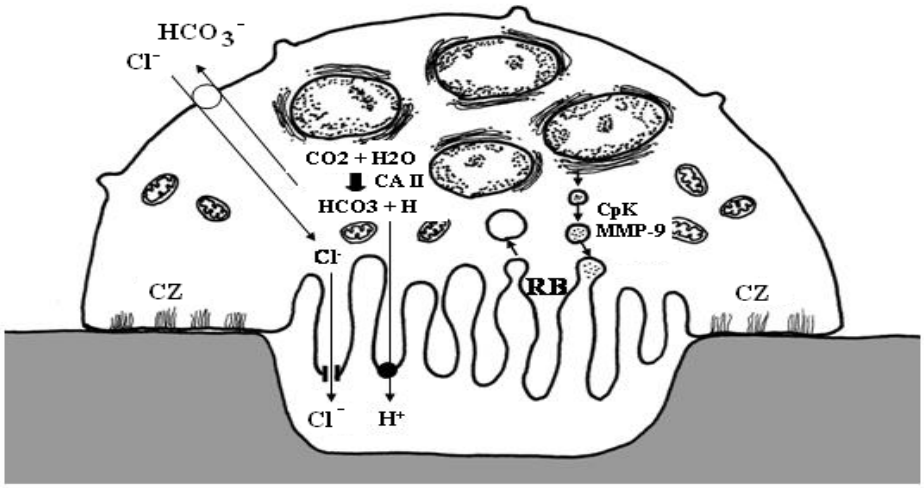

Fig. 3 Mechanism of osteoclast bone resorption.
Osteoclast secretes proton produced by carbonic anhydrase II (CA II) into Howship's lacuna through vacuolar type H^+-ATPase. Chloride ions are also transported into the lacuna by chloride channel. The acidic environment dissolves hydroxyapatite in bone matrix. On the other hand, organic components such as collagen are degraded by cathepsin K (CpK) and matrix metalloproteinase (MMP) -9. RB:ruffled border, CZ; clear zone

mitochondria, endoplasmic reticulum and well-developed Golgi apparatus around nuclei. They also contain vesicles, lysosomes, tubular lysosomes and vacuoles [1, 2]. These structures indicate that osteoclasts are actively involved in energy production and protein synthesis, particularly production of lysosomal enzymes.

Active bone-resorbing osteoclasts show definite cell-polarity. Their plasma membrane is classified into three regions: clear zone, ruffled border, and basolateral plasma membrane.

Clear zone was named by clear appearance lacking cell organelle. This region shows a ring-like structure and consists of accumulated focal contacts. Cytoplasm of clear zone contains abundant actin filaments (Fig. 2B). Actin ring observed in *in vitro* osteoclasts by phalloidine staining correspond to this structure. Clear zone is engaged in attachment of osteoclast to bone matrix and isolation of bone-resorbing compartment from extracellular fluid. This compartment provides efficient condition for bone resorption. The attachment of osteoclast to the bone matrix is mediated by vitronectin receptor, $\alpha_v\beta_3$ integrin, in membrane of clear zone[28]. One of the ligands is considered to be osteopontin in bone matrix. Cell-matrix interaction stimulates c-Src, a non-receptor-type tyrosine kinase, involving in maintenance of cell polarity and activity of osteoclasts. Soriano et al. [29] revealed that c-Src-deficient mice showed a phenotype of osteopetrosis. Despite numerous osteoclasts, they do not develop ruffled border, indicating that c-Src is essential for bone resorbing function of osteoclasts. c-Src appears to control cytoskeleton by acting with Pyk2, a focal adhesion kinase [30], and c-Cbl, a proto-oncogene [31]. Additionally, coated pits indicating receptor-mediated endocytosis are occasionally seen in clear zone. MT-MMP1 is also localized in clear zone [32]. These findings suggest that clear zone is also involved in endocytosis of bone matrix and migration of osteoclasts.

Prominent feature of osteoclast is ruffled border, the folding of the plasma membrane in the area facing bone matrix (Fig. 2C). Ruffled border is closely associated with bone resorption. Bone resorption is achieved by dissolution of mineral components consisting of hydroxyapatite and degradation of organic

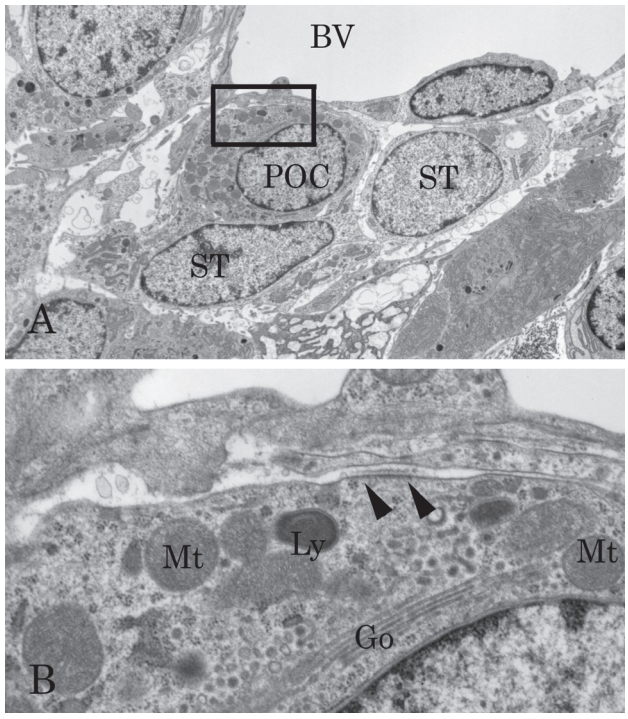

Fig. 4 Electron micrographs of a preosteoclast.
A: A preosteoclast is surrounded by stromal cells (ST). BV; blood vessel
B: Electron micrograph, at a higher magnification, of square in A. The preosteoclast possesses numerous mitochondria (Mt), the Golgi apparatus (Go) around a nucleus, and lysosomal structures (Ly). Adherent structures through extracellular matrices and close contact structures (arrows) are seen at the region between the preosteoclast and the stromal cell.

components of bone matrix. Carbonic anhydrase, converting CO_2 and H_2O into H^+ and HCO_3^-, in cytoplasm [33, 34] and vacuolar type H^+-ATPase in ruffled border membrane are involved in acidification of Howship's lacuna [35] (Fig. 3). H^+-ATPase is composed of 13 subunits, forming a large complex. Oc/oc mice showing osteopetrosis attribute to the abnormality of the subunit of H^+-ATPase[36]. Numerous mitochondria in osteoclasts are considered to provide ATP for H^+ transport. Thus, extracellular acidic environment (pH 4-5) under ruffled border leads to focal decalcification of hydroxyapatite in bone matrix. Moreover, ion balance in cytoplasm is maintained by chloride channel (CIC)-7. Importance of this channel is also clarified by ClC-7-deficient mice showing osteopetrosis [37].

In contrast, extracellular digestion of organic components is accomplished by lysosomal enzymes such as cathepsin K [38, 39] and MMP-9 [18]. Cathepsin K belongs to a cysteine protease family and could degradate collagen fibers in acidic condition. Therefore, this protease mainly participates in degradation of native collagen in bone matrix. In fact, immunolocalization of cathepsin K is detected in Howship's lacunae. On the other hand, MMP-9 acts as a gelatinase for further digestion of segmented collagen fibrils (Fig. 3). Tartrate resistance acid phosphatase (TRAP) is widely used as a marker enzyme of osteoclasts and secreted in Howship's lacunae. Although this enzyme could dephosphorylate osteopontin [40], its precise role in bone resorption has not been clarified yet.

Basolateral plasma membrane of osteoclasts is thought to be responsible for receiving stimulation of calcitonin and cytokines. This area is also important for cell-cell interaction with osteoblast-lineage cells. Salo et al. [41] divide basolateral membrane into the central region and the lateral region. They consider the former region is a functional secretory domain involved in transcytosis, exocytosis of degradated bone matrix [41, 42].

Calcitonin is a hormone to inactivate osteoclasts. This causes destruction of actin filaments, loss of clear zone and retraction of osteoclast, followed by detachment from bone surface. These processes are also caused by dibutyryl cAMP and increase of cytoplasmic calcium, indicating that protein kinase A and C may regulate activity of osteoclasts through cytoskeletal reorganization [43, 44]. In addition, extracellular signal-regulated kinase (ERK) is also involved in disorder of cytoskeleton by calcitonin [45]. Thus, it is conceivable that ERK participates in the maintenance of cell polarity of osteoclasts as well as their survival [46, 47].

Differentiation of osteoclasts

It is widely accepted that osteoclasts originate from monocyte-macrophage lineage precursor cells. Because osteopetrotic animals cured by bone marrow transplantation or parabiosis are linked by a common crossing circulation with normal littermates[1]. This evidence suggests that osteoclast precursor cells were carried through blood capillaries and resided in bone tissue. Osteoclast precursors or preosteoclasts show several resemblances in morphological feature to osteoclasts. They possess numerous mitochondria, the Golgi apparatus around nucleus and lysosomal structures (Fig. 4). They also express TRAP, cathepsin K and calcitonin receptors. Osteoclast precursors demonstrate undifferentiated characteristics such as a large amount of free ribosome and a few rough endoplasmic reticulum. Nevertheless, it is difficult to identify undifferentiated osteoclast precursors by their morphological characteristics because sections only reveal a limited aspect of them.

Molecular mechanism of osteoclast differentiation and activation has been clarified. First finding was a discovery of macrophage colony stimulating factor (M-CSF) as a critical factor for osteoclast differentiation. Osteopetrotic (op/op) mice showing a marked reduction of osteoclasts in bone tissue were caused by a point mutation of the M-CSF gene. Their osteopetrotic phenotypes were cured by administration of recombinant M-CSF [48]. Currently, M-CSF is required for proliferation of osteoclast precursors and differentiation and survival of osteoclasts. However, M-CSF is not enough to induce osteoclast differentiation in vitro. Osteoclasts appear in bone tissue of op/op mice according to their age. These results suggest that other factors would be also required for osteoclastogenesis.

Morphological findings have demonstrated that osteoclasts and preosteoclasts always come in contact with ALP-positive osteoblast-lineage cells. It had been suggested that cell-cell interaction between osteoblast-lineage cells and osteoclast precursors is essential for osteoclastogenesis [49, 50]. One of the most exciting findings was the discovery of RANK/RANKL system in osteoclast differentiation [51, 52]. RANKL, a member of the tumor necrosis factor (TNF) family, was originally reported to be expressed in activated T cells. RANKL, produced in osteoblast-lineage cells, participates in differentiation and activation of osteoclasts via binding to RANK, expressed in osteoclast progenitors and osteoclasts. The critical role of RANK/RANKL system was confirmed by mouse genetic studies. RANK- or RANKL-deficient mice show osteopetrosis attributing to the defect in osteoclastogenesis. Stimulators of osteoclastogenesis such as $1,25\text{-}(OH)_2 D_3$, prostaglandin E_2 (PGE_2), interleukin (IL)-1, PTH and PTH related protein upregulate the expression of RANKL in osteoblast-lineage cells. Conversely, OPG, a soluble form of the TNF receptor, works as a decoy receptor for RANKL and inhibits osteoclastogenesis. OPG-deficient mice demonstrate severe osteoporosis associated with an increased number of osteoclasts [53]. OGP expression is upregulated by estrogen, TGF-β and BMP. Thus, differentiation and activation of osteoclasts are controlled by the balance between RANKL and OPG in osteoblast-lineage cells. The effects of hormones and cytokines converge on RANKL and OPG [54].

Signaling of RANK/RANKL in osteoclast-lineage cells is mediated by TNF receptor-associated factor (TRAF). TRAF6 is associated with intracellular domain of RANK. TRAF6-deficient

mice with osteopetrosis revealed that this molecule is implicated in osteoclast differentiation as well as activation [55, 56]. Although TRAF6 stimulates ERK, JNK, p38 and NF-κB signalings, genes regulated by them had not been fully understood. Recently, microarray regarding RANKL-inducible genes revealed that NFATc1 [57, 58] and dendritic cell-specific transmembrane protein (DC-STAMP) are involved in osteoclast differentiation. DC-STAMP-deficient mice demonstrate that mononuclear osteoclastic cells instead of multinucleated osteoclasts could resorb bone matrix [59]. On the other hand, FcRγ and DAP12 harboring immunoreceptor tyrosine-based activation motif (ITAM) cooperate with RANK to stimulate calcium signaling and activate NFATc1 [60]. Thus, signaling pathway involving in immunoresponse also regulates osteoclast differentiation. This new research area, referred to osteoimmunology, might unveil the mechanism of pathological bone resorption.

We reported cell attachment structures between osteoclast-lineage cells and osteoblast-lineage cells [61]. Extracellular matrix, including heparan sulfate proteoglycan and fibronectin, is involved in cell attachment. In addition to RANK/RANKL system, other regulatory mechanism such as cell-cell attachment might be important to determine the region where osteoclasts should differentiate because osteoclasts always appear in bone tissue. Future research will be necessary to understand a signaling pathway mediated by this cell-cell interaction.

Conclusion

Bone remodeling is performed by osteoblasts and osteoclasts. Their proliferation, differentiation and function are regulated by hormones such as parathyroid hormone (PTH), estrogen, $1,25(OH)_2D_3$ and calcitonin as well as cytokines. In the case of osteoblasts, bone morphogenetic protein (BMP) is one of the most effective cytokines. On the other hand, osteoclastogenesis requires M-CSF and RANKL. It is no doubt that cell-cell interaction between osteoblast-lineage cells and osteoclast-lineage cells is essential for maintenance of bone volume. Recent bone research including discovery of Runx2 and RANK/RANKL system provide progress for understanding bone metabolism. However, it is not enough to explain the mechanism of bone remodeling. Interdisciplinary research including cell biology, biochemistry, physiology, morphology and etc. will be necessary to clarify the bone cell biology and develop therapy for bone diseases such as osteoporosis and periodontal disease.

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