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BONE REMODELING BY OSTEOCLASTS: WHAT HAVE WE LEARNED FROM GENE KNOCKOUT STUDIES?

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SUMMARY – Osteopetroses are disorders of bone remodeling resulting in increased bone mass. Osteopetrosis abnormalities can include changes in osteoclast lineage, bone marrow microenvironment, or both. Little is known about the mechanisms that regulate the activity of different bone cell types. Various agents act on bone in a complicated web of interactions, with either synergistic or diverse effects. Advances in molecular biology have enabled studies in knockout or transgenic animals, providing an insight into the mechanism of bone remodeling.

Key words: *Osteopetrosis, etiology; Osteoclasts, physiology; Bone remodeling, genetics; Animal*

Introduction

Three major cell types are involved in skeletogenesis: (1) osteoblasts, which are the main producers of specialized matrix that undergoes mineralization; (2) chondrocytes, which form cartilage, but some of them are further involved in bone formation; and (3) osteoclasts, which are involved in bone resorption. These cells are permanently active while bone remodeling occurs continuously throughout the lifetime of a body.

Osteopetroses are disorders of bone remodeling characterized by impaired osteoclast function, which results in a net increase of skeletal mass. Osteoclastic defects in osteopetrosis could result from abnormalities within the osteoclast lineage itself (hematopoietic), from defects in the microenvironment, or both.

Little is known about the molecular mechanisms that regulate the activity of different bone cell types. Recently, studies utilizing transgenic (overexpression of certain genes) and knockout (lacking certain genes) mice have provided an insight into the mechanism of action of different factors involved in the regulation of bone formation and remodeling. Osteopetrotic mouse is an excellent model for investigation of the molecules involved in the osteoclast development and function of cells and factors involved in bone remodeling.

Osteoclasts and Their Origin

Osteoclasts are multinucleated cells of hematopoietic origin, derived from precursors common to osteoclasts and monocyte-macrophages, found only in bone[1,2]. It is likely that resorptive cells are derived from the colony forming unit – granulocyte-macrophage (CFU-GM). *In vitro* experiments have shown that, under the influence of osteoclastogenic factors, stem cells differentiate into bone resorptive cells. Direct contact between osteoclast precursors and osteoblasts or stromal cells is indispensable for osteoclast differentiation, while osteoclastogenesis-modulating cytokines are produced in the bone microenvironment by marrow stromal cells, immature monocytes, osteoblasts and their precursors, acting mainly on osteoclast precursors and causing the release of other cytokines in a paracrine fashion[1]. *In vitro* experiments have further demonstrated that, in the presence of osteoblasts or stromal cells, even mature macrophages and monocytes can differentiate into osteoclasts[3].

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The prominent features of osteoclasts as well as of macrophages are lysosomes and mitochondria in the cytoplasm. Osteoclasts, however, exert an extracellular phagocytic activity, showing polarity and forming a 'ruffled border'. Ruffled border is present only in the membrane area near the bone surface with a lot of deep invaginations and projections from which vacuoles bud off and to which lysosomes fuse. Ruffled border is surrounded by a clear zone that lacks organelles, by which the osteoclasts are attached to the bone surface. The ruffled border and clear zone form a great secondary extracellular lysosome between bone and osteoclast[4].

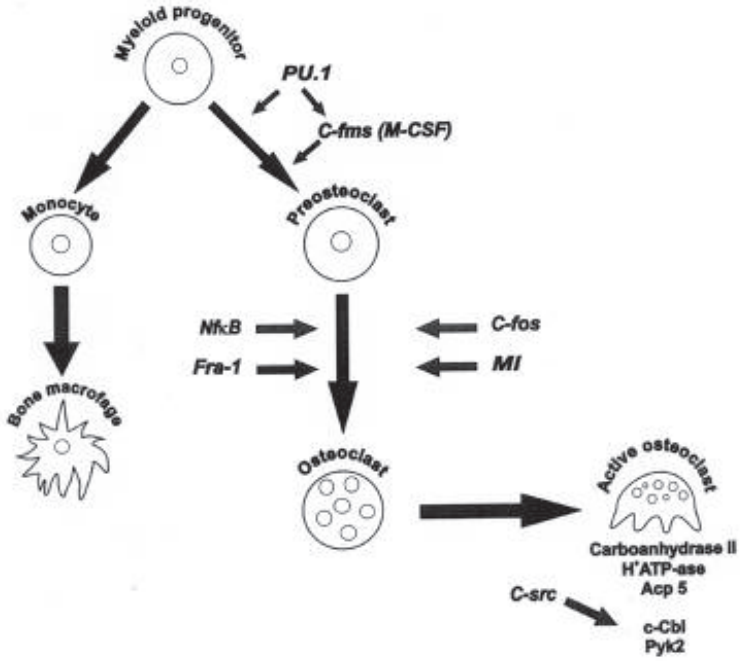

Fig. 1. Osteoclast differentiation. Differentiation from myeloid precursor cells is under control of PU.1 and M-CSF. PU.1 also stimulates transcription of c-fms that encodes M-CSF receptor, while M-CSF, along with Nf-κB, mi and c-fos, is responsible for osteoclast maturation. Functional mature osteoclast is the result of c-src, c-Cbl, Pyk2, cathepsin K and acid-phosphatase involvement.

Osteoclasts acidify the extracellular compartment facilitating in this way the dissolution of calcium phosphate hydroxyapatite and action of several proteinases (serine protease, tartarate-resistant acid phosphatase (TRAP), cathepsin K, matrix metalloproteinase-9 (MMP-9)). Acidification is achieved by the presence of a unique osteoclast isoenzyme, vacuolar type H+ ATPase. Carbonic anhydrase II is present in osteoclasts as a cellular source of protons, deficiency of which leads to osteopetrosis, renal tubular acidosis (RTA), and cerebral calcifications[4].

Essential step in bone resorption is digestion of type I collagen. As many *in vitro* experiments have shown, one of the key enzymes in bone remodeling is MMP-9 (gelatinase B), a member of the MMP family, which degrades most components of extracellular matrix[5,6]. MMPs could be obligatory for osteoclast migration to the resorption sites, since *in vitro* experiments have shown the migration of preosteoclast cells to be completely blocked by MMP inhibitors. MMP-9 is produced in osteoclasts and released into the subosteoclastic space during resorption of bone matrix. MMP-9 expression was significantly stronger in osteoporotic bones compared to normal controls, suggesting an important role in excessive resorption[5]. In multiple myeloma, tumor cells produce abundant MMP-9 (and MMP-2) that could be responsible for infiltration and osteolysis of bone[7]. The expression pattern and localization of MMP-9 in physiologic and pathologic conditions suggest a key role in the bone resorbing process[8-10].

Gene Knockout Studies

Advances in molecular biology and biotechnology have enabled determination of gene function by its overexpression (transgenic animals) or by studying animals lacking certain genes (knockout experiments)[11,12].

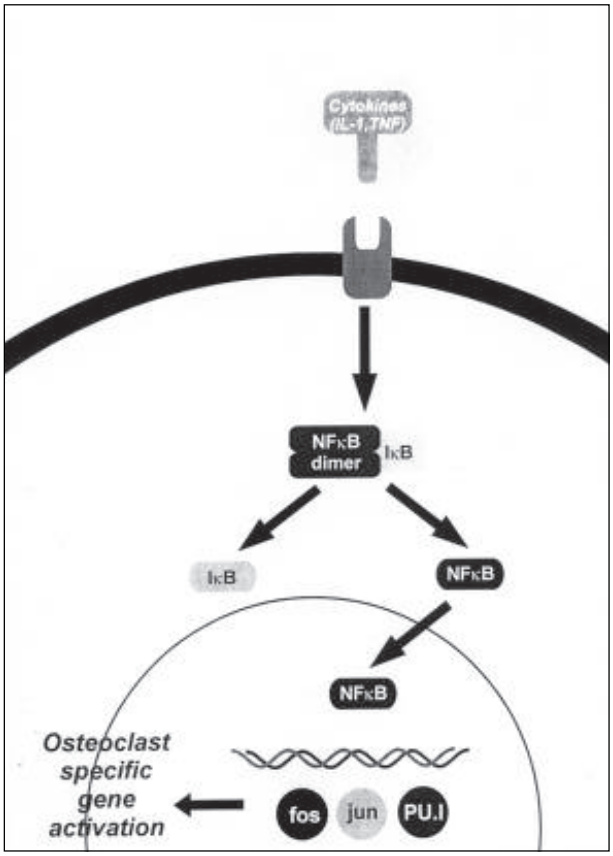

Fig. 2. Nf-κB is a set of five transcriptional factors which form dimers and are bound in cell cytoplasm to inhibitory proteins IκB (α,β,ε). Once cells are stimulated, IκB is degraded, allowing NF-κB to enter the nucleus and activate specific gene transcription.

Several genes have been found to have an important role in the pathogenesis of osteopetrosis mediating osteoclast differentiation and function (Fig. 1).

C-fos

C-fos is a nuclear proto-oncogene, a member of the Fos family. All members of the Fos family (which includes fosB, fra-1 and fra-2) are able to associate with the Jun protein family members[13,14]. C-fos is one of the components of the activator protein-1 (AP-1) transcription factor, a dimeric DNA binding protein[15]. Constitutive c-fos expression is seen in a limited number of tissues: fetal liver, amniotic and placental tissue, adult bone marrow, growing bone and developing central nervous system[13,16].

Overexpression of c-fos in transgenic mice results in dysregulation of bone growth, and in forming osteosarcomas and chondrosarcomas[17-19]. Although expressed in numerous tissues, c-fos knockout mice (-/-) develop severe osteopetrosis as a dominant phenotype. In other cells, other members of the Fos or Jun family probably compensate for c-fos[17].

It seems that in bone, c-fos exerts its effect on bipotential macrophage/osteoclast precursors, because mutant animals completely lack functional osteoclasts and their immediate precursors, while the number of bone marrow macrophages is increased relative to the wild type mice. In other tissues, the macrophage count is normal. Fos mutant microenvironment is capable of supporting normal hematopoiesis, while the mutant phenotype can be rescued by bone marrow transplantation from normal littermates[17,18,20].

In vitro, hematopoietic cells of fos -/- mice fail to develop into osteoclasts. Treatment with c-fos antisense in coculture had negative effect on osteoclast differentiation, but had no effect on bone resorbing activity after osteoclasts have already formed. In addition, infection of these hematopoietic precursors with c-fos-expressing retrovirus restores their osteoclastogenic capacity[21].

Fra-1

Fra-1 (Fos related antigen 1) is a nuclear proto-oncogene, also member of the Fos related family of proteins. It encodes protein that shares extensive amino acid homology with Fos, has leucine zipper domain and C-terminal region that is conserved in the fos gene family[22,23]. Fra-1 is involved in early transcription cell response to extracellular stimuli and, like Fos (and all members of the Fos family), binds cooperatively with Jun to form AP-1[24,25]. Interestingly, in cells of Fos lacking mice, gene transfer of Fra-1 protein was most efficient of four Fos proteins (c-Fos, FosB, Fra-1 and Fra-2) in rescuing osteoclast differentiation block. Furthermore, overexpression of c-Fos in immortalized precursor cell lines did not enhance osteoclastic differentiation, while Fra-1 increased substantially the number of osteoclast-like cells. These data suggest that Fra-1 has a role in osteoclast differentiation that cannot be substituted by c-Fos and that it could be crucial for regulation of AP-1 activity, which is essential for osteoclast formation. Although c-Fos is essential for osteoclast formation, Fra-1 could be the factor needed for full responsiveness of osteoclast precursors[26,27].

C-src

C-src is a normal cellular gene. It encodes a 60 kd tyrosine kinase localized on the cytoplasmic side of the plasma membrane. The c-src proto-oncogen product, pp60c-src, seems to play a vital role in impaired bone re-

sorption because pp60c-src-/- mice develop osteopetrosis, and neither of the src-related proteins (like c-fyn, c-lyn) can compensate for pp60c-src in osteoclasts[28-30]. A gene derived from c-src, called v-src, is responsible for the formation of sarcoma of viral etiology. In mouse, src is expressed in all cells at variable levels. The highest level of expression is in platelets and neuron growth cones, where it is found in two different forms, one of which displays higher kinase activity. Src family kinases associate with c-fms (receptor for colony stimulating factor 1 (CSF-1)), and both src and c-fms kinases may be involved in a common signal transduction in osteoclasts. Src family members may be able to compensate for each other in many cell types. This, however, is not the case in osteoclasts[31]. How c-src controls osteoclast function, is not fully understood. Evidence that there are downstream proteins in c-src signaling pathway, such as c-Cbl and Pyk2, has been recently presented. Both c-Cbl (proto-oncogene product) and Pyk2 (pyruvate kinase 2) are phosphorylated by src kinase and are required for osteoclast adhesion and bone resorption[32-35].

In c-src knockout (-/-) animals, the osteoclast lineage is intact but the cells are functionally impaired and are unable to resorb bone. In addition, src activity could be involved in the regulation of cathepsin-K, cathepsin-L and cathepsin-B proteases that are essential in degrading the organic phase of bone[36].

C-src -/- mice develop osteopetrosis, have shorter bones with partial absence of bone marrow, and their incisors fail to erupt. Though osteoclasts in bone marrow can be identified, resorption is minimal to absent, and on electron microscopic examination osteoclasts from c-src nullmutant mice contain no ruffled membrane. *In vitro* experiments have shown that osteoclasts from src mutants fail to form bone resorption pits, suggesting that the lack of bone resorption is intrinsic to osteoclasts and not reflective of the bone microenvironment[31,37,38].

Op/op

Animals with op/op mutation fail to produce competent macrophage colony stimulating factor (M-CSF), and show a failure in generating monocytes, macrophages and osteoclasts[39]. M-CSF is one of the cytokines required for normal osteoclast formation and is normally produced by fibroblasts, uterine epithelial cells and by the decidual layer of placenta. It provides a signal required for the survival, proliferation and maturation of cells with osteoclast characteristics, as shown in many *in vitro* experiments[40,41].

In op/op mice, both fibroblasts and osteoblasts are defective in producing active M-CSF. Its mRNA is present at normal levels, but the protein is aberrant. The op mutation maps to a single base pair insertion in the coding region of the M-CSF gene, resulting in insertion of a stop codon 21bp downstream producing a nonfunctional protein. op/op fibroblasts produce only granulocyte-macrophage colony stimulating factor (GM-CSF)[42,43]. This results in a severely reduced number of monocytes, tissue macrophages and osteoclasts. Osteoclasts that do differentiate are larger, but their function is impaired[44]. Studies showed that daily injections of M-CSF in mice corrected cell count of affected cell lines. However, the number of macrophages in the ovary, uterus and synovia did not increase suggesting existence of differentiation pathways other than CSF-1 dependent one[44].

The microenvironment in which these cells develop is defective[45]. It is likely that other cytokines compensate for the lack of active M-CSF, as mutant mice recover by week 22[46-48].

Mi/mi

Osteopetrotic microphthalmia (mi/mi) mutant mice have a number of defects, e.g., loss of pigmentation, decreased number of mast cells, deafness, reduced eye size, and impaired bone resorption[49-51]. Mutations in *mi* gene in humans are associated with Waardenburg's syndrome type 2, an inherited disorder characterized by hearing loss and impaired pigmentation[50,51]. The *mi* gene encodes a member of basic-helix-loop-helix leucine zipper (bHLH-ZIP) family of transcription factors called mitf (microphthalmia transcription factor). Two mutations of *mi* gene produce altered proteins that do not bind DNA and act as negative alleles in osteoclasts leading to osteosclerosis[50]. Osteoclasts from mutant animals differentiate normally but are significantly smaller with smaller clear zones and abnormal ruffled border[52]. There are fewer normal, multinucleated osteoclasts, while the number of less efficient, mononucleated cells is increased[53,54].

Granulocyte Macrophage – Colony stimulating Factor (GM-CSF -/-)

GM-CSF was defined by its ability to stimulate colonies of granulocytes and macrophages in cell cultures. However, it acts on many cell types including hematopoietic stem cells, megakaryocytes, erythroid progenitors and eosinophils[55]. *In vitro* studies showed a significant increa-

se in TRAP (tartarate resistant acid phosphatase, a marker of osteoclasts) positive cell count in response to GM-CSF treated progenitors, significant even when compared to M-CSF treated progenitors[56,57]. Given intravenously, murine GM-CSF caused leukocytosis in peripheral blood and had a myeloproliferative effect in rat bone marrow, but its effect in marrow was lesser as compared to M-CSF or G-CSF alone[58]. Interestingly, in GM-CSF nullmutant mice, skeleton develops normally and osteoclast differentiation occurs, suggesting that the action of GM-CSF may thus be compensated by another cytokine *in vivo*. Possible candidates include interleukines -1 and -3 (IL-1, IL-3) and tumor necrosis factor (TNF)[59-61].

PU.1 -/-

PU.1 (Spi-1, Sfpi-1) is a specific transcription factor regulating expression of many genes in myeloid and B-cells[62], with a very important role in hematopoiesis[63,64]. In myeloid cells, PU.1 regulates transcription of c-fms and Mac1 proteins that are central to the monocyte phenotype. PU.1 is, together with c-Fos, essential for differentiation into osteoclast lineage[65-67]. Expression of PU.1 parallels induction of osteoclastogenesis *in vitro* after treatment with 1.25(OH)2D3 and dexamethasone, and is expressed throughout the temporal span of osteoclastogenesis[68].

Animals mutant for PU.1 have mature erythrocytes, megakaryocytes and T cells but no mature myeloid or B cells, and die from septicemia within 2 days following delivery[62,69]. These animals also develop severe osteopetrosis in all bones including vertebrae, with cartilaginous bars persisting deeply in metaphyseal bone. PU.1-/- mice are devoid of osteoclasts as well as of macrophages in the marrow, lungs and liver. Mutant animals can be saved by marrow cell transplantation, suggesting that this mutation is a cell autonomous defect in a common hematopoietic progenitor and that PU.1 transcription factor regulates initial steps of myeloid differentiation[67,68].

NF-κB -/-

The NF-κB transcription factor family regulates the expression of series of target genes that are involved in development and immunity, in regulation of cell proliferation and apoptosis and response to stress[70-72]. The major form of NF-κB is a heterodimer that is formed from two of five proteins p50, p52, p65 (RelA), Rel B and Rel C[70]. It is retained in cytoplasm by receptor molecules such as IκB family (IκB*α,β,ε*) as well as NF-κB precursors p105 (NF-Jκ B1) and p100 (NF-κB2)[73-76]. After cell stimulation, IκB is phosphorylated by Ikappakinases (IKK-1, 2) and degraded allowing NF-κB to translocate into the nucleus[77,78]. Exceptions are mature B-cells where NF-κB is a constitutive nuclear protein[77].

NF-κB1 and NF-κB2 functions are required for normal B cell and osteoclast/macrophage development, and are probably involved at a similar step as c-fos. NF-κB -/- animals fail to generate mature osteoclasts and B-cells, which leads to severe osteopetrosis and impaired host defense[71,79]. NF-κB proteins are prominent in common signal transduction pathways of the osteoclastogenic cytokines such as IL-1, IL-6 and TNF-*α*[80]. Interestingly, NF-kB upregulates back IL-1, IL-6 and TNF gene expression, so the once activated effect of cytokines could be enormously amplified[70]. This could also explain why deletion of an individual cytokine does not significantly affect osteoclast differentiation. It appears that other cytokines may compensate for this defect using this common pathway[71,80-82]. The role of NF-κB in survival and bone resorption activity of osteoclasts has been confirmed in a series of *in vitro* experiments[83,84] (Fig 2).

Cathepsin-K

Cathepsin-K is a cystein protease, present in osteoclasts, which is secreted into the subosteoclastic space and is essential for degradation of the organic phase of bone[85-90]. Mutations in cathepsin-K gene in humans lead to pyknodysostosis, an inherited disorder characterized by acroosteolysis of distal phalanges, short stature, skull deformities and impaired osteoclast function causing osteosclerotic changes in bone[85,91]. Cathepsin K deficient mice are osteopetrotic, with disturbed hematopoiesis and splenomegaly[86]. Osteoclasts derived from knockout mice are fully differentiated, but have undefined resorptive surface containing undigested collagen fibrils[85,86]. The function of these cells is severely impaired while they are incapable or resorbing type I collagen, an important step in the process of bone resorption[92,93].

Acp 5

Tartarate-resistant acid phosphatase 5 is an enzyme specifically expressed in osteoclasts and is used as a marker of osteoclast lineage. Mice lacking osteoclast tartarate-resistant acid phosphatase (Acp 5 -/-) develop a mild form of osteopetrosis. Animals are healthy, develop normally and have normal reproductive capacity. Characteristics of Acp5 knockout are skeletal deformities that become more apparent with age: shortened and deformed long bones, shortened vertebrae, expanded growth plates with disor-

ganized loci of bone modeling and ossification and increased mineralization. Osteoclast function in mutant mice is impaired. Interestingly, resorption alveolar crest is active enough to allow normal dentition.

Mutation occurs on chromosome 9 where a single Acp 5 gene is located, and leads to osteoclast cell-intrinsic defect of resorption activity. This enzyme is apparently required for normal modeling and remodeling of developing and adult skeleton[94].

Conclusion

Bone remodeling is a process maintained in fragile balance under the influence of numerous hormones, growth factors and cytokines. Numerous studies on osteopetrotic animal mutants provide valuable information about the etiology and pathophysiology of osteopetrosis, which is a highly heterogeneous disorder. Although correlation between animal models and human diseases is limited, studies on knockout animals provide an insight into the bone biology in general. This knowledge of various factors that influence bone modeling and remodeling enable us to understand processes in bone metabolic disorders and to develop different strategies in treating them. However, there are still questions that have to be answered, since for two mouse osteopetrotic mutations, i.e. osteosclerotic (oc/oc) and gray lethal (gl), the mutated genes have not yet been identified.

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Sažetak

KOŠTANA PREGRADNJA POMOĆU OSTEOKLASTA: ŠTO SMO NAUČILI IZ ISPITIVANJA *KNOCKOUT* GENA?

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Osteopetroze su poremećaji pregradnje kostiju koji dovode do povećanja koštane mase. Osteopetrotične abnormalnosti mogu obuhvaćati promjene u staničnoj liniji osteoklasta, u mikrookolišu koštane srži ili oboje. Malo se zna o mehanizmima koji reguliraju aktivnost različitih vrsta koštanih stanica. Različiti čimbenici utječu na kost kroz čitav splet međusobnih aktivnosti sa sinergističnim ili raznovrsnim učincima. Napredak u molekularnoj biologiji omogućio je ispitivanja na *knockout* ili transgenskim životinjama, pružajući uvid u mehanizme koštane pregradnje.

Ključne riječi: *Osteopetroza, etiologija; Osteoklast, fiziologija; Koštana pregradnja, genetika, Životinja*