Mutation in Osteoactivin Enhances RANKL-Mediated Signaling, Promoting Osteoclast Differentiation, Survival and Inhibiting Bone Resorption

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Introduction: Osteoactivin (OA/Gpnmb) was first identified by differential gene display in mutant osteopetrotic rats (1). The novelty of OA/Gpnmb in bone regeneration has been characterized and reported by our group (2,3). Recent study demonstrated that OA/Gpnmb is highly expressed by osteoclasts in vitro (4). The same group showed that targeted overexpression of OA/Gpnmb in cells of the osteoclastic lineage demonstrated the importance of OA/Gpnmb in osteoclast activity and bone resorption (5). To specify the critical role and mechanism of action of OA/Gpnmb in osteoclastogenesis, we characterized the osteoclastic phenotype in a mouse model with natural mutation in the OA/Gpnmb gene. We hypothesized that mutation of OA/Gpnmb increases osteoclast formation and decreases its activity.

Methods: For the purpose of the study, we used a genetically modified D2J mouse model at age of 8-week. D2J mice are mutant for OA/Gpnmb, characterized with nonsense mutation with early stop codon, generating a truncated protein sequence of 150 a.a. The wild-type (WT) is D2J/Gpnmb+ that have been generated by knocking in OA/Gpnmb alleles into D2J mouse. Mouse femurs were evaluated by μ-CT, histology and histomorphometric analyses. Mice sera were examined by ELISA for bone resorption markers; collagen I C-peptide (CTX-1), RANKL and OPG. Bone marrow-derived hematopoietic stem cells (BM-HSCs) isolated from mouse femurs and tibiae were differentiated into osteoclasts using RANKL and M-CSF for 7 days. Osteoclast differentiation was evaluated by TRAP activity and staining. Osteoclast resorptive function was examined using corning disc and human bone chip osteoassays. For osteoclast survival, cells were cultured for two additional days in the presence of RANKL only. Markers of osteoclast differentiation were tested by quantitative (q)PCR SYBR green. MAPK and AKT signaling pathways in osteoclast were examined by Western blot analysis. Statistical analysis in this study was carried out by student-t-test.

Results: Micro-CT analysis of D2J femoral diaphyses show marked decrease in bone surface and perimeter as well as total cortical porosity in comparison to D2J/Gpnmb+ WT mice (Figure-1). In contrast, cortical thickness of D2J is significantly increased compared to D2J/Gpnmb+ WT mice, probably due to defective osteoclast function. Serum ELISA of CTX-1 is significantly lower in D2J, however TRAP and RANKL/OPG ratio are not different, compared to D2J/Gpnmb+ WT mice. Although histomorphometric analysis show no difference in TRAP-positive osteoclast surface and number, however, the eroded surface, number and depth of resorption pits are significantly decreased in D2J compared to D2J/Gpnmb+ WT (Figure-2). Moreover, ex vivo differentiation of D2J osteoclasts on corning discs and bone chips show reduced resorption area, decreased calcium and CTX-1 in condition medium, respectively. Transmission electron microscopy micro-images of D2J osteoclasts show loss of polarization with short and scanty ruffled border over the bone surface in comparison to D2J/Gpnmb+ WT osteoclasts. In ex vivo cultures, number of TRAP-positive multinucleated osteoclasts (≥3 nuclei) as well as their surface area are substantially increased in D2J compared to D2J/Gpnmb+ (Figure-3). Quantitative PCR analysis show temporal increased gene expression of TRAP, DC-Stamp, RANK receptors, c-Fos, NFATc1, cathepsin K and calcitonin receptors in D2J osteoclasts compared to D2J/Gpnmb+. Additionally, in ex vivo cultures, multinucleated osteoclasts of D2J show increased ability to survive in the presence of RANKL due to delayed onset of apoptosis. RANKL signaling is enhanced in D2J multinucleated osteoclasts as determined by increased phosphorylation of ERK, P38 and JUNK in MAPK pathway. Most interestingly, AKT and GSK-3β phosphorylation in D2J multinucleated osteoclasts is upregulated, suggesting that mutation of OA/Gpnmb results in increased osteoclast survival and decreased bone resorption.

Discussion: In this study, mutation of OA/Gpnmb increased osteoclast differentiation confirmed by increased TRAP-positive osteoclasts ex vivo, an effect mediated by enhanced RANKL signaling through MAPK pathway. In addition, OA/Gpnmb gene mutation improved osteoclast survival, an effect mediated by increased AKT phosphorylation. Interestingly, OA/Gpnmb mutation inhibited osteoclast function in bone resorption confirmed by lower CTX-1 in sera and bone chips. Cumulatively, these in vivo and ex vivo results show that, on one hand, OA/Gpnmb negatively regulates osteoclast differentiation, survival, and signaling events, whereas on the other hand, it positively influences osteoclast function. Future research studies will focus on evaluation of the osteoclast phenotype in ovariecetomed D2J mice. We will also emphasize on the rescuing effect of recombinant OA/Gpnmb protein on osteoclast differentiation and function, in vivo and in vitro.

Significance: Inhibiting bone resorption is one of the classical methods for treating osteoporosis, a skeletal disease characterized by low bone mass with significant deterioration in the bone micro-architecture leading to high bone fragility. The identification of novel factors that regulate osteoclastogenesis and gaining insights into their mechanisms of action, will promote the development of new therapeutic modalities aiming to increase bone mass and prevent bone loss.

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References:

Figure 1. μCT analysis of femurs in 8-wks D2J and wild-type D2J/Gpnmb+ (A) 3D μCT reconstruction of the sagittal planes of 1 mm femoral diaphysis of D2J and D2J/Gpnmb. (B) Cortical thickness (Ct. Th) and total porosity were calculated. Data presented in panel (B) represent mean ±SEM in 4 mice 8-weeks old. c: p< 0.001
Figure 2. Histomorphometric analysis of femurs in D2J and D2J/Gpmnb+. (A) Femur sagittal sections showing TRAP positive osteoclasts on the trabecular bone in D2J and D2J/Gpmnb+ mice. High Mag. show D2J femur sections with few osteoclasts under periostium. Osteoclasts localized on trabecular bone surfaces were flattened in shape and more vacuolated with undefined resorption pits in D2J compared to D2J/Gpmnb+. (B) Total number of osteoclasts (N.Oc) per bone perimeter (B.Pm) and eroded surface (ES) over bone surface were calculated. Data presented in panel (B) represent Mean ± SEM in 5 mice per genotype group. b: p< 0.01. Arrows; TRAP+ve osteoclasts. Asterix; periostium.
Figure 3 Differentiation of bone marrow-derived hematopoietic stem cells (BM-HSC) into osteoclasts from D2J. BM-HSC from D2J and D2J/Gpnmb+ were primed with MCSF and differentiated with RANKL then cultures were fixed for TRAP staining and activity. (A) Microscopic pictures of TRAP positive osteoclasts showing larger osteoclasts in D2J. Asterisk refers to osteoclast size. (B) Total number of TRAP positive osteoclasts (N.Oc) with nuclei ≥3 with nuclei (3-19, 20-49 and >50) at RANKL of 20 ng/mL. Osteoclast surface area (Oc. Sr.Ar) was calculated at RANKL (R) dose of 10, 20 and 50 ng/mL. Data in panel (A) and (B) represent Mean ±SEM in 6-wells per experiment. Experiment was repeated 6 times and showed similar pattern. Asterisk, size of osteoclast.