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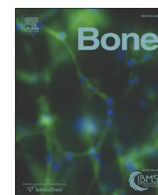

Original Full Length Article

MKP-1 signaling events are required for early osteoclastogenesis in lineage defined progenitor populations by disrupting RANKL-induced NFATc1 nuclear translocation☆,☆☆

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ABSTRACT

Cytokine-directed osteoclastogenesis is initiated in response to *m*acrophage *c*olony *s*timulating *f*actor (M-CSF) and *r*eceptor *a*ctivator of *NF-κB l*igand (RANKL) to drive formation of osteoclasts (OC), large bone resorptive cells of hematopoietic origin. RANKL-induced signaling activates the MAPK pathways, which initiates nuclear translocation of the master regulator of osteoclast formation, transcription factor NFATc1. Proper control over these signaling events is essential to normal OC formation response to stimuli. MAPK phosphatase 1 (MKP-1), a serine and tyrosine phosphatase encoded by the gene *Dusp1*, functions to dephosphorylate and subsequently inactivate MAPK (p38 and JNK) signaling essential in osteoclastogenesis. Here, we explored the role of MKP-1 during RANKL-driven osteoclastogenesis from defined (B220/CD45$^-$GR1$^-$CD11b$^{lo/-}$CD115$^+$) OC progenitor (dOCP) populations using WT and *Dusp1*$^{-/-}$ global knockout mice. Sorted cells were driven to OC by M-CSF pre-treatment followed by RANKL stimulation for 3 days. OC formation and qPCR products were analyzed for maturation. Results indicate that *Dusp1*$^{-/-}$ dOCP form less numerous, significantly smaller and less functional OC compared to WT controls. These data were corroborated by mRNA expression of the key OC genes, *Nfatc1* and *Tm7sf4* (DC-STAMP), which were significantly reduced in early osteoclastogenesis in OC progenitor from *Dusp1*$^{-/-}$ mice. Intriguingly, our data reveals that MKP-1 may positively control OC formation in response to RANKL by regulating NFATc1 nuclear translocation. Collectively, this report supports the idea that MKP-1 signaling is essential in early osteoclastogenesis in response to RANKL-induced signaling.

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Introduction

Bone turnover and remodeling require the formation of osteoclasts (OCs), large multinucleated cells with the capacity to resorb bone matrix [1–5]. Bone marrow-derived OCs arise from hematopoietic stem cell (HSC) progenitor populations late in monocyte differentiation and have been distinctly defined as CD45R$^-$GR-1$^-$CD11b$^{lo/-}$CD115$^+$ (defined here as defined OCPs or dOCPs) [6–8]. One of the key elements to OC formation is the ability of cells to respond to cytokines that drive their differentiation. Macrophage-colony stimulating factor (M-CSF), a cytokine that stimulates survival, primes these cells for lineage commitment to either a macrophage or to a pre-OC by increasing expression of the RANK receptor [4,9,10]. Receptor activator of NF-κB ligand (RANKL), produced by proximal osteoblasts/stromal lineage cells and lymphocytes within the bone microenvironment, signals a temporal process through the RANK receptor where progenitors begin lose their macrophage-like phenotype and develop into multinucleated, active OCs, approximately 72-h post RANKL exposure through modulation of signal transduction pathways within the pre-OCs [11].

At the sub-cellular level, RANKL-induced signaling activates transcription factors (TFs) NF-κB and AP-1 (c-fos/c-Jun) through phosphorylation of pathway intermediates, IκB (NF-κB) or p38 and JNK MAPK, respectively [12,13]. These intermediates direct the recruitment, dephosphorylation and translocation of nuclear factor of activated T cell (NFATc1), the master regulator of osteoclastogenesis in response to RANKL [5,14,15]. NFATc1 controls OC formation through transcriptional regulation where NFATc1 oligomerizes with other TFs and binds to promoter regions of osteoclast-specific genes including; *Nfatc1*, tartrate resistant acid phosphatase (TRAP/*Acp5*), dendritic cell-specific transmembrane protein (DC-STAMP/*Tm7sf4*), calcitonin receptor (*CR*) and cathepsin K (*Ctsk*) [16].

While several signaling pathways are engaged during osteoclastogenesis, the precise mechanisms controlled by the signaling events are

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poorly understood. Distal RANKL-induced signaling events include activation of the MAPK and NF-κB signaling cascades whose activity is controlled through kinases and phosphatases. One family that controls MAPK phosphorylation is the *du*al-specific *s*erine *p*hosphatases (*Dusp*), which function to dephosphorylate serine and tyrosine residues [17–19]. *Dusp1*, the family archetype encodes for MAP Kinase Phosphatase-1 (MKP-1), that preferentially dephosphorylates phospho(p)-p38 and p-JNK. We have reported that in response to periodontal pathogen constituents, including bacterial lipopolysaccharide (LPS), MKP-1 ablation leads to enhanced and sustained activation of the MAPK, contributing to an inflammatory phenotype and bone loss [20,21] and forced expression of MKP-1 reduces inflammation and bone loss in LPS-induced models. Together, these reports support the assertion that MKP-1 functions to negatively regulate inflammatory driven OC formation through signaling pathways including p38 and JNK MAPK. However, while much is known about the role of MKP-1 in LPS-driven OC formation, little is known about the role of MKP1 signaling in the RANKL-driven systems.

Given their common lineage, most studies have evaluated OC formation in progenitors defined by the macrophage lineage ($CD11b^+$) [22]. In contrast, further isolation of enriched, dOCP populations, may uncover subtle differences in OC progenitors within the bone marrow HSC population [6]. Our objective in the present study was to delineate the role of MKP-1 in RANKL-stimulated osteoclastogenesis from defined OC progenitor populations. Based on previous observations which show that MKP-1 is a negative regulator of OC formation in response to LPS challenge, we hypothesized that MKP-1 will also negatively regulate RANKL-induced OC formation. Surprisingly, we report that MKP-1 deletion resulted in fewer, smaller and less functional OCs in defined OCPs following RANKL stimulation. These results provide evidence for MKP-1 signaling as a permissive regulator of OC formation in response to RANKL, likely through interaction between the MAPK family and NFATc1 activation.

Materials & methods

Animals

Mixed genetic background B6:129P WT and *Dusp1*$^{-/-}$ mice were obtained from Bistol-Myers Squibb Pharmaceutical Research Institute and bred at the Medical University of South Carolina Animal Facility and maintained in accordance with NIH guidelines. Mice were subject to food and tap water ad libitum and maintained under normal 12-hour light cycles. The Institutional Animal Care and Use Committee (IACUC) at the Medical University of South Carolina approved all subsequent experimental protocols.

Selection of osteoclast progenitor populations

Osteoclastic cell progenitor populations were isolated from WT and *Dusp1*$^{-/-}$ female bone marrow using lineage-specific and progenitor markers as $B220/CD45^-GR1^-CD11b^{lo/-}CD115^+$ as previously defined [6]. Briefly, hematopoietic stem cells (HSCs) were enriched initially by using the suspension populations after 24 h of whole marrow culture. Using the AutoMACS sorter (Miltenyi Biotec), bone marrow HSCs were sorted into three distinct groups based on expression of CD11b: $CD11b^{hi}$ (macrophage lineage), $CD11b^{lo}$ ($dOCP^{lo}$) and $CD11b^-$ ($dOCP^-$). Defined osteoclast progenitor (dOCP) populations were immunophenotyped using commercially available antibodies from Miltenyi Biotec (Auburn, CA) including anti-B-cell lineage (mAb): anti-CD45R/B220 (RA3-6B2), anti-T-cell lineage (mAb): anti-CD3e (17A-2), anti-granulocyte lineage (mAb): anti-Gr-1/Ly-6G (R86-8C5), anti-monocyte/macrophage lineage (mAb): anti-CD11b (M1/70) and anti-monocyte progenitor antibody (mAb): anti-cfms/CD115 (AFS98; R&D Systems, Minneapolis, MN). All antibodies were directly conjugated fluorochromes or biotinylated from the manufacturer. Isolated cells were labeled for flow cytometric analysis following standard procedures. Dead cell exclusion was performed using propidium iodide (PI) staining. Flow cytometry analysis was performed on at least 30,000 events per sample using the Miltenyi MACSQuant (Auburn, CA). Data analysis was performed using the MACSQuant Software provided with the machine.

Osteoclast differentiation assays

$CD11b^{hi}$ and dOCP populations were plated in 96-well plates at a density of 5×10^4 cells/well in priming media (10 ng/ml M-CSF) overnight. Once seated, cells stimulated with fresh control media (25 ng/ml M-CSF) or treatment media (25 ng/ml M-CSF and 50 ng/ml RANKL) for two days. Fresh control and treatment media were replaced every two days and used to stimulate cells for 24 h, for a total of 3 days of exposure to RANKL. Treated cells were fixed with 10% glutaraldehyde and TRAP stained as previously described [23]. For proof of principle, OC formation assays described above were repeated in the presence of p38 (SB508250) and JNK (SP600125) inhibitors. Cells were treated with inhibitors 30 min prior to stimulation with M-CSF and RANKL. An investigator blinded to the sample identity quantified osteoclasts based on the following criteria; 1) number of OCs per field of view, 2) number of nuclei per OC (≥3) and 3) size of OC. OCs were measured using Image J software. In addition, the density, distribution and the percentage of TRAP positive cells using 0–3 arbitrary scales were evaluated (0 indicated low density/no $TRAP^+$, up to 3 indicating density greater than 75% of the well and highly $TRAP^+$). To measure the OC activity, sorted OCPs were seeded onto bovine cortical bone slices and treated with M-CSF and RANKL as described previously. Bone slices were harvested eight-days following initial priming to allow OC function to occur. OC function was measured following cell removal with a 5% hypochlorite solution for 10 min and then counterstaining lacunae with using 0.1% Toluidine blue solution in water. Resorption lacunae were enumerated based on field of view at 20× magnification under an inverted microscope. As a secondary surrogate measure of OC function, the pNPP-based TRAP enzyme activity assay kit was utilized according to the manufacturer's protocol (Takara Bioscience, Japan) at the day of harvest. Activity was measured by absorbance at 405 nm and normalized to total protein.

Immunoblotting

Sorted dOCP populations were plated on 35 mm dishes at 2×10^6 cells/dish. Cells were primed with M-CSF (10 ng/ml) for two days prior to stimulation. Cells were serum starved with 2% serum-supplemented α-MEM for 2 h, then treated with RANKL (100 ng/ml) for indicated time points. Cells were lysed by RIPA buffer and quantified using the Lowry assay (BioRad, Hercules, CA). 20 μg of total protein was loaded onto 10% TGX gels (BioRad, Hercules, CA) and electrophoresed. Separated proteins were then transferred onto nitrocellulose membranes using the wet-transfer method and blocked in 5% milk for 1 h prior to probing with rabbit-anti-mouse derived antibodies including; phospho(p)-p38, total p38, p-JNK (p46/54), total JNK, p-ERK (p42/44), total ERK and GAPDH. All primary antibodies were diluted in 5% BSA (in TBS-T) and incubated at 4 °C overnight. Anti-rabbit and anti-biotin secondary antibodies, each conjugated to HRP, was used to visualize the blots in the presence of West Pico ECL (Pierce).

RT-qPCR

mRNA was isolated from M-CSF or M-CSF/RANKL treated cells, three days following stimulation (1×10^6 cells/well) using the TriZOL reagent (Invitrogen) according to the manufacturer's specifications. Total extracted mRNA was quantitated using Nanodrop spectrophotometry (BioRad, Hercules, CA). cDNA was synthesized using TaqMan reverse transcriptase reagent kit (Applied Biosystems, Foster City, CA) and 500–1000 ng of total RNA. Amplified cDNA was then probed using amplicon primers obtained from Applied Biosystems including; RANK

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