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Ubiquitin-Like Domain of IKK β Regulates Osteoclastogenesis and Osteolysis

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Abstract

The transcription factor NF- κ B family is central for osteoclastogenesis and inflammatory osteolysis. Activation of NF- κ B dimers is regulated by a kinase complex predominantly containing IKK α (IKK1), IKK β (IKK2), and a regulatory subunit, IKK γ /NEMO. IKK α and IKK β catalyze the cytoplasmic liberation and nuclear translocation of various NF- κ B subunits. The requirement of IKK α and IKK β for normal bone homeostasis has been established. Congruently, mice devoid of IKK α or IKK β exhibit in vitro and in vivo defects in osteoclastogenesis, and IKK β -null mice are refractory to inflammatory arthritis and osteolysis. To better understand the molecular mechanism underlying IKK β function in bone homeostasis and bone pathologies, we conducted structure-function analysis to determine IKK β functional domains in osteoclasts. IKK β encompasses several domains, of which, the ubiquitination-like domain (ULD) has been shown essential for IKK β activation. In this study, we examined the role of ULD in IKK β -mediated NF- κ B activation in osteoclast precursors and its contribution to osteoclastogenesis and osteolysis. We generated and virally introduced IKK β in which the ULD domain has been deleted (IKK β Δ ULD) into osteoclast progenitors. The results show that deletion of ULD diminishes IKK β activity and that IKK β Δ ULD strongly inhibits osteoclastogenesis. In addition, unlike wild-type (WT)-IKK β , IKK β Δ ULD fail to restore RANKL-induced osteoclastogenesis by IKK β -null precursors. Finally, we provide evidence that IKK β Δ ULD blocks inflammatory osteolysis in a model of murine calvarial osteolysis. Thus, we identified the ULD as crucial for IKK β activity and osteoclastogenesis and that ULD-deficient IKK β is a potent inhibitor of osteoclastogenesis and osteolysis.

Keywords

IKK β ; Ubiquitin; Osteoclast; Osteolysis

Introduction

Osteoclasts are the predominant cells responsible for bone resorption and their bone resorptive activity is essential for maintaining bone homeostasis [1, 2]. However, certain

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genetic impairments, metabolic flaws, and environmental insults may negatively impact bone homeostasis by altering osteoclast activity. Among the most common disparities are age-related hormonal imbalance and inflammatory responses [3, 4]. Therefore, understanding the mechanisms governing osteoclast development and activity have been and continue to be the focus of research efforts combating bone loss ailments.

In addition to its crucial role in osteoclast development and function [5], the transcription factor family NF- κ B has emerged in recent years as central mediator of inflammatory responses and osteolysis [4, 6, 7]. At the center of this family is the IKK complex comprised of the kinases IKK α , IKK β and the scaffold protein IKK γ /NEMO [8]. The IKK complex forms in response to activation of transmembrane receptors by certain extra cellular stimuli. In this regard, inflammatory stimuli, such as LPS and TNF as well as the osteoclastogenic ligand RANKL activate their respective receptors leading to recruitment and activation of the IKK complex which in turn phosphorylates downstream inhibitory molecules leading to their proteosomal degradation. As a result, various NF- κ B subunits, including p50, p52, p65, RelB, and c-Rel are released, shuttled to the nucleus where they activate transcription.

The role of NF- κ B subunits and kinases in skeletal development has been described. Whereas combined deletion of p50 and p52 subunits of NF- κ B arrested osteoclastogenesis and resulted in severe osteopetrosis, single deletions of others proteins in the pathway were mostly consistent with varying degrees of phenotypic severity [9–11]. Based on these studies, several approaches targeting NF- κ B signal transduction were developed to alleviate inflammatory osteolysis. In this regard, short peptides that inhibit NEMO oligomerization and others designed to inhibit NEMO binding to IKK α and IKK β , successfully blocked bone erosion and osteolysis in various animal models of experimental arthritis and calvarial osteolysis [12–16]. However, we recently reported that constitutively active IKK β (i.e. IKK β SSEE) induced cytokine-independent osteoclastogenesis in vitro and osteolysis in vivo [17, 18]. Remarkably, this action was refractory to treatment with the above mentioned inhibitory and decoy peptides, suggesting that inhibitory strategies targeting IKK β -proximal moieties, including NEMO and receptor-ligand interactions, are ineffective in this case. Therefore, the need for molecular approaches targeting IKK β directly is essential. In this regard, we have recently reported that intact tyrosine residues 188/199 are absolutely essential for activation of IKK β [19]. In fact mutating these two tyrosine residues was sufficient to attenuate the constitutive activity of IKK β SSEE. In the current study, we targeted yet another regulatory element in IKK β , namely the ubiquitin-like domain (ULD) of IKK β . To this end, we provide evidence that deletion of ULD attenuates the kinase activity of IKK β and IKK β SSEE. Consistently, ULD-mutated IKK β /IKK β SSEE failed to reconstitute the osteoclastogenic potential of IKK β -null precursor cells, effectively inhibited osteoclastogenesis by wild type cells, and markedly blocked inflammatory osteolysis in mice.

Material and Methods

Reagents

Antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA) and Cell Signaling Technologies, Inc. (Danvers, MA). Chemicals were purchased from Sigma (St. Louis, MO). pMx retroviral expression plasmid was from Dr. T. Kitamura (University of Tokyo, Japan). Mouse cDNA for IKK β was purchased from ATCC.

Animals—Wild-type C57BL/6 mice at 5–6 weeks of age were used. All mice were housed in a controlled barrier facility at Washington University (St. Louis, MO). Generation of IKK β -null mice was previously described [20].

Generation of Retrovirus—All expression constructs were subcloned into pMx using standard techniques. Generation of the various IKK β deletion/mutant constructs was previously described [18]. The use of Plat-E retrovirus packaging cells stably expressing retroviral structural proteins gag-pol and env for transient production of high titer retrovirus was described previously [21]. Briefly, 8 μ g pMx vectors expressing gene of interest were transfected into 5 million plat-E cells (grown in DMEM supplemented with 10% FBS and penicillin/streptomycin) using Fugene 6 (Roche, Palo Alto, CA) according to manufacturer's instructions. Twenty-four hrs post transfection, media was exchanged to remove transfection reagent. Twenty-four and 48 hrs post media exchange, supernatant was collected and pooled for transduction of monocytes.

Generation of Adenovirus—Adenovirus expressing IKK β was generated by subcloning from the pMx parental vector into Ad5 CMV K-NpA Shuttle using EcoR1 and Not1 restriction endonucleases (New England Biolabs, Ipswich, MA). Recombination [22], production and characterization (pfu/particle) of virus was provided by Viraquest, Inc. (North Liberty, IA). For local *in vivo* gene transfer in mice, 1 $\times$ 10⁷ pfu of virus diluted in 50 μ l sterile PBS were injected over the supracalvarially. Six mice were utilized for these experiments with comparable results. LPS (10 μ g) diluted in 50 μ l total volume with PBS was injected supracalvarially. *In vivo* injections were executed with 1.00 cc insulin syringes with 29G needles. Seven days post-injection calvaria were fixed, decalcified, and analyzed histologically for osteoclasts and osteolysis.

Generation of Monocyte/Macrophages (osteoclast progenitors)—Marrow was flushed from long bones into alpha-Minimum Essential Medium (MEM). Cell pellets were resuspended in whole media (α -MEM with 1X penicillin/streptomycin, 10% heat-inactivated fetal bovine serum (FBS)). Monocytes/macrophages were produced by growing cell suspensions in the presence of 10 ng/ml M-CSF. Monocytes/macrophages were allowed to proliferate for 3 days at 37 °C in 5% CO₂ at which point they were infected with retrovirus (50% virus supernatant, 50% α -MEM containing 10% FBS, 10ng/ml M-CSF, penicillin/streptomycin, and 4 μ g/ml hexadimethrine bromide). Twenty-four hrs post transduction, cells were selected in α -MEM containing 10% FBS, 10ng/ml M-CSF, penicillin/streptomycin, and 2 μ g/ml puromycin for 72 hrs, at which point selection media was removed, cells were washed, and grown for 24 additional hrs without puromycin. At this point, cells were lifted, counted, and plated per experimental conditions.

In vitro Osteoclastogenesis—3 $\times$ 10⁴ monocytes were plated in 200 μ l α -MEM with 10% FBS per well. IKK β expressing cells were cultured in 10ng/ml recombinant mouse M-CSF (R&D Systems), plus recombinant mouse 100ng/ml RANKL (R&D Systems) for 4 days. At this point, cells were fixed and TRAP stained using the Leukocyte Acid Phosphatase Kit (Sigma, St. Louis, MO). TRAP-positive cells with three or more nuclei were scored as osteoclasts.

Kinase Assay—Plat E cells expressing indicated flag-tagged IKK β constructs were lysed in immunoprecipitation (IP) buffer (50 mM Tris (pH 8), 120 mM NaCl, 0.5% NP-40, 50 μ g/ml leupeptin, 10 μ g/ml aprotinin, 50 μ g/ml phenylmethylsulfonyl fluoride (PMSF), 0.2 mM sodium orthovanadate, 100 mM sodium fluoride). IKK β was immunoprecipitated with M2 antibody, washed twice with IP buffer, once with kinase assay buffer (Cell Signaling Technologies Danvers, MA), and incubated for 30 minutes at 30°C in 30 μ l kinase assay buffer with 1 μ g GST-I κ B α , 2.5mM MgCl₂, and 16 μ M ATP. Reaction was terminated with 30 μ l reducing sample buffer. Samples were analyzed by Western blot.

Nuclear Isolation and Electrophoretic Mobility Shift Assay (EMSA)—Cells were resuspended in hypotonic lysis buffer A (10 mM HEPES [pH 7.8] 10 mM KCl, 1.5 mM MgCl, 0.5 mM dithiothreitol 0.5 mM 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), 5 µg/ml Leupeptin) and incubated on ice for 15 min, and NP-40 was added to a final concentration of 0.64%. Nuclei were pelleted and the cytosolic fraction was carefully removed. The nuclei were then resuspended in nuclear extraction buffer B (20 mM HEPES[pH 7.8] 420 mM NaCl, 1.2 mM MgCl, 0.2 mM EDTA, 25% glycerol, 0.5 mM dithiothreitol, 0.5 mM AEBSF, 5 µg/ml Pepstatin A, 5 µg/ml Leupeptin), vortexed for 30 sec and rotated for 30 min in 4°C. The samples were then centrifuged and the nuclear proteins in the supernatant were transferred to fresh tubes and protein content was measured using standard BCA kit (Pierce, Rockford, IL). A portion of the nuclear extracts (10 µg) was incubated with an end-labeled double stranded oligonucleotide probe containing the sequence 5'- AAA CAG GGG GCT TTC CCT CCT C -3' [23] derived from the κ B3 site of the TNF promoter. The reaction was performed in a total of 20 µl of binding buffer (20 mM HEPES [pH 7.8], 100 mM NaCl, 0.5 mM dithiothreitol, 1 µg poly dI-dC, and 10% glycerol) for 30 min at room temperature. Samples were then fractionated on a 4% polyacrylamide gel and visualized by exposing dried gel to film. Another portion of the nuclear extracts was mixed with sample loading buffer and subjected to Western blot to detect p65 nuclear expression.

Histology—*Calvarial* bones were collected from C57bl/6 mice experimental groups and fixed in 10% buffered formalin for 24 hours. Bones were then decalcified for 7 days in buffer consisting of (14% (w/v) EDTA, H4NOH pH 7.2), dehydrated in ethanol (30%–70%), cleared through xylene, and embedded in paraffin. Sections were stained histochemically for TRAP to visualize osteoclasts. Immunohistochemistry was performed according to manufacturer's instructions for immunoperoxidase staining.

Microscopy—Cells and histological sections were imaged under white or ultraviolet light on an inverted microscope (Olympus IX-51). Digital images were captured using a CCD camera (Olympus DP70, 12 mega-pixel resolution).

Statistics—Results are representative of at least four independent experiments with similar findings. ANOVA was conducted for comparison between means of various treatment groups. Results are reported as *p* values.

Results

IKK β Δ ULD is a dominant- negative inhibitor of NF- κ B

IKK β is crucial for activation of NF- κ B. We recently described that constitutive activation of IKK β in myeloid cells causes spontaneous osteoclastogenesis in vitro and leads to the development of pathologic osteolysis in mice [17]. Therefore, we examined the functional role of IKK motifs to regulate the function of this kinase. To this end, we cloned full-length (wild type) and ULD-deleted forms of IKK β (referred to as IKK β Δ ULD) in a retroviral plasmid (pMx) to enable transduction in osteoclast precursors (fig 1A). First, we confirmed that both constructs are retrovirally expressed in osteoclast precursors (fig 1B). Consistent with previous findings [24, 25], we found that deletion of the ubiquitin-like domain in IKK β reduced the catalytic activity of the kinase (fig 2A-B) evident by failure of IKK β Δ ULD to induce activation of its downstream target p65, and failure to phosphorylate I κ B in vitro. In contrast, WT-IKK β was able to phosphorylate I κ B and lead to nuclear accumulation of p65 as expected. Consistent with these results, using gel shift mobility assay, we also document that whereas WT-IKK β induced DNA binding activity of NF- κ B in response to RANKL within 10 minutes, no such binding was observed in the presence of IKK β Δ ULD (fig 2C),

suggesting that this mutated form of IKK β is a potent dominant negative inhibitor of NF- κ B activation.

IKK β Δ ULD inhibits osteoclastogenesis, in vitro

NF- κ B is crucial for osteoclast differentiation and survival. We and others have shown that IKK β plays a crucial role in this process and that deletion of IKK β from early osteoclast progenitors impedes osteoclastogenesis [20, 26]. Therefore, we examined the role of IKK β Δ ULD in osteoclastogenesis. To this end, progenitors were transduced with WT-IKK β or IKK β Δ ULD. Super-repressor I κ B (SR- I κ B), which we have demonstrated previously that it acts as a potent dominant-negative inhibitor of osteoclastogenesis [27, 28], was included as a control. The results depicted in figure 3 show that whereas WT-IKK β elevates (also refer to panel B), IKK β Δ ULD strongly inhibits osteoclastogenesis. The latter response was indistinguishable from the expected dominant negative effect of the SR- I κ B.

To further examine the negative osteoclastogenic effect of IKK β Δ ULD we utilized IKK β -null (KO) precursor cells. These cells do not generate osteoclasts [20]. We transduced these IKK β - null cells with GFP, WT-IKK β , catalytically-deficient IKK β (IKK β -KM), constitutively active IKK β (IKK β -SSEE), or IKK β Δ ULD. Cells were then treated with RANKL and M-CSF for 4 days and osteoclastogenesis was assessed. The results (fig 4A-B) indicate that WT-I κ B restored osteoclastogenesis by null cells to levels comparable with wild type cells. The catalytically-dead kinase failed whereas the constitutively active kinase restored osteoclastogenesis higher than wild type levels, as we expected. Consistent with its anti-osteoclastogenic role, IKK β Δ ULD failed to restore osteoclasts by IKK β -null precursors.

IKK β Δ ULD blocks inflammatory osteolysis in mice

NF- κ B pathway mediates inflammatory and osteolytic responses. Our previous work has shown that inhibition of NF- κ B at various stages attenuates murine osteolysis [12, 13, 16, 27, 29, 30]. Thus, based on our in vitro observations, we surmised that IKK β Δ ULD is a promising tool to inhibit inflammatory osteolysis. To test this possibility, lipopolysaccharide (LPS) injections (10 μ g) were administered locally over the calvaria in the presence of vehicle or adv- IKK β Δ ULD. As expected, LPS caused a severe osteolytic response (fig 5) evident by generation and recruitment of mass numbers of TRAP-positive osteoclasts and subsequent bone erosion (arrows). Consistent with its proposed anti-osteolytic role, virally-produced IKK β Δ ULD completely blocked the LPS-mediated osteolytic response (fig 5A-B) and significantly reduced the number of osteoclasts at the resorptive site (fig 5C).

Discussion

The osteoclastogenic roles of various molecules in the NF- κ B pathway have been described. Furthermore, it was shown that components of the classical NF- κ B pathway, dominated by IKK β , as well as components of the alternative NF- κ B pathway contribute to osteoclast formation and activity [4, 9, 31]. In addition, the classical NF- κ B pathway mediates wide range of inflammatory pathways [26, 32, 33]. Indeed, ample evidence point to a central role of IKK β in inflammatory bone diseases and position this kinase as an attractive target for osteolytic bone therapies.

The most compelling evidence for IKK β regulation of osteolysis was evident from our recent discovery that constitutively active IKK β heightened osteoclastogenesis in vitro and in vivo, and caused severe bone loss in transgenic mice [17, 18]. This effect of constitutively active IKK β was independent of proximal stimuli including RANKL, TNF and IL-1 signaling. Even more surprisingly, the osteoclastogenic action of this active form of IKK β

was independent of proximal NF- κ B component most notably its immediate partners IKK α and NEMO. In fact, administration of NEMO-binding domain decoy peptide, which we have demonstrated previously as potent inhibitor of NF- κ B and osteoclasts, failed to impact the activity of constitutively active IKK β .

The potent IKK β independent osteoclastogenic effect was further verified in vivo. In this regard, using tissue-specific knock-in approach, we expressed the constitutively active form of IKK β in the myeloid lineage. Mice harboring this IKK β form presented significant bone loss [17].

The observations described herein suggest that inflammatory osteolysis secondary to constitutively active IKK β may be refractory to therapeutic modalities aimed at proximal RANK/RANKL and NF- κ B signaling targets. Therefore, there is a need to explore IKK β intrinsic regulatory elements to modulate its activity. In this regard, we reported recently that tyrosine phosphorylation of IKK β at residues Y188/Y199 is a prerequisite for its activation and that mutation of one or both tyrosine residues renders IKK β inactive [19]. More importantly, this mutant form of IKK β acts as a dominant negative inhibitor of NF- κ B and osteoclastogenesis.

The ubiquitin-like domain of IKK β was first reported as negative regulator of its kinase activity [24]. In the present study, we examined the physiological relevance of IKK β -ULD. We show that deletion of ULD abolishes the kinase activity of IKK β in vitro evident by its inability to phosphorylate its immediate target protein I κ B α . Consequently, ULD-deficient IKK β not only fail to activate NF- κ B but also acts as a dominant-negative inhibitor blocking activation of endogenous IKK β in response to RANKL stimulation of marrow macrophages. Given the established role of IKK β in osteoclastogenesis, we examined the effect of IKK β Δ ULD in this setting. Consistently, IKK β Δ ULD inhibited osteoclastogenesis as efficiently as SR-I κ B did. In addition, unlike the wild type and constitutively active forms of the kinase, IKK β Δ ULD failed to restore osteoclastogenesis when introduced into IKK β -null cells treated with RANKL, just as the kinase-dead form, IKK β -KM, failed.

Finally, the functional relevance of IKK β Δ ULD was tested in vivo, using an inflammatory osteolytic mouse model that we have established previously. Specifically, we have tested the potential anti-osteolytic activity effect of virally-produced IKK β Δ ULD in LPS-induced murine calvarial osteolysis. The findings clearly support the notion that IKK β Δ ULD is a potent inhibitor of inflammatory osteolysis and present a potential therapeutic utility. It should be noted, however, that the short duration of the experiment (one week) does not permit definitive conclusion as to potential adverse effects. Given that the ULD peptide alone was reportedly sufficient to inhibit TNF-induced NF- κ B activity [24], we anticipate that administration of this short peptide, similar to NBD, will be more effective owing to lesser potential immunogenicity and easier application.

The exact mechanism by which ULD inhibits NF- κ B and osteoclastogenesis remains unclear. However, the overall findings point to inactivity of IKK2. This inactivity does not stem from failure to assemble an IKK complex but possibly due to interference with the activation T loop following conformational change. This is somewhat supported by our unpublished observations whereby deletion of ULD ablates the kinase activity of the constitutively active form of IKK2, namely IKK2SSEE. Further experiments are required to establish the underpinning mechanism.

Signal-induced mono-ubiquitination of IKK2 at lysine 163 was previously described by Carter and colleagues [34, 35]. This report also describes regulation of phosphorylation of the activation T loop by ubiquitination. The physiological significance of this lysine 163 ubiquitination in osteoclastogenesis and inflammatory osteolysis, if any, remains unclear.

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Figure 1.

ULD-mutated IKKβ (IKKβΔULD) is virally expressed in osteoclast precursor cells. A) Stick figure of wild type and IKKβΔULD with relevant domain depiction. B) Wild type and IKKβΔULD were virally produced. Viral particles were added to osteoclast precursors and their respective protein expression was measured in equal cell lysates by Western blots using anti IKKβ antibody.

Figure 2.

IKK β Δ ULD is catalytically inactive and does not activate NF- κ B. IKK β -null osteoclast precursors were infected with retroviral (pMx) WT-IKK β or ULD-deleted IKK β form. Cells were then treated with RANKL as indicated. Cytosolic and nuclear fractions were isolated and subjected to Western blots to detect input material (β -actin) and nuclear p65 expression, respectively (A). Cells as described in panel A were immunoprecipitated with anti-IKK β antibody. The immunoprecipitate was then subjected to in vitro kinase assay using recombinant GST-I κ B as a substrate to demonstrate in vitro phosphorylation (B). Nuclear extracts isolated from same cells described in panel B, were subjected to NF- κ B electrophoretic mobility gel shift assay (C). n.s. = non-specific binding.

Figure 3. IKK β Δ ULD inhibits osteoclastogenesis. A) Osteoclast precursors were infected with control (pMx alone), WT-IKK β , IKK β Δ ULD, or super-repressor I κ B (SR-I κ B). Cells were then cultured in the presence of RANKL and M-CSF for 4 days, fixed and TRAP-stained. B) Osteoclast were counted from quadruple wells and three independent experiments representative of panel A. Statistical analyses comparing IKK β Δ ULD with control and SR-I κ B with control have *P* value <0.05 for both.

Figure 4.

IKK β Δ ULD does not restore osteoclastogenesis by IKK β -null precursor cells. IKK β -null osteoclast precursors (KO) were infected with retroviral particles expressing GFP, WTIKK β , IKK β SSEE (constitutively active), IKK β -KM (kinase mutant), and IKK β Δ ULD. Wild type (WT) cells infected with GFP were used as control. Cells were then plated in the presence of M-CSF and RANKL for 4 days, fixed, and TRAP-stained to detect osteoclasts (arrows). *P* value <0.05; comparing 1) KO+IKK β SSEE group with KO+GFP, and 2) KO +IKK β Δ ULD with KO+IKK β SSEE group.

Figure 5.

IKK β Δ ULD is a potent inhibitor of calvarial inflammatory osteolysis. Adenoviral particles of IKK β Δ ULD (1×10^7 pfu) and LPS ($10 \mu\text{g}$) were administered over the calvaria of mice separately or in combination at day 0 and every two days thereafter. Calvaria were collected on day 7 and processed for immunostaining (A) and histomorphometric (B-C) analysis. S - Indicates midsagittal suture. Arrows point to osteolysis and osteoclasts (red staining). Control mice were injected with PBS. Panel B represents the average of osteolytic area (μm^2) from three independent experiments measured using Osteomeasure software [36]. *P* value *, ** < 0.005 compared as indicated by lines. C) Number of osteoclasts per surface

area as described previously [36] representing the average of three independent experiments. *P* values * <0.005, ** <0.01.