The role of PI3Kβ and PLCγ2 proteins in osteoclasts and bone homeostasis

PhD Theses

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INTRODUCTION

Due to the increasing prevalence of age-related bone diseases in the Western countries, there is an urgent need for the better physiological and pathophysiological understanding of the cellular and molecular mechanisms underlying pathological bone loss. Osteoclasts are the unique bone-resorbing cells of hematopoietic origin, which are critically involved in the maintenance and repair of the mammalian skeleton. Their development is directed primarily by M-CSF and RANKL cytokines as well as by adhesive interactions. Mature osteoclasts are polarized cells that form actin ring structures to develop a tight connection with the bone surface, where they secrete hydrochloric acid and lytic enzymes into the resorption pit. To erode underlying bone, acidification of the resorption compartment by the hydrochloric acid, and solubilisation of the bone matrix by lytic enzymes - such as cathepsin K - is carried out. The detailed physiological understanding of the pathogenesis of bone loss by osteoclasts may lead to novel strategies for the treatment of chronic bone diseases, including rheumatoid arthritis and postmenopausal osteoporosis.

Phosphoinositide 3-kinases (PI3K) are a diverse family of lipid kinases phosphorylating phosphoinositide moieties at position 3 of the inositol ring. The best known mammalian PI3-kinases are the Class I PI3-kinase family members PI3Kα, PI3Kβ, PI3Kγ and PI3Kδ. Prior

studies suggested important roles for PI3K activity in osteoclast biology. It was shown first by Nakamura and his colleagues, that the general PI3K inhibitor wortmannin inhibits the development and resorptive activity of osteoclasts. Though the above study indicated an important role for PI3Ks in osteoclast biology, it is still unclear which isoform mediates the various functions of PI3Ks in those cells. The above issues prompted us to analyze the role of PI3Kβ in *in vitro* osteoclast development and function as well as in *in vivo* bone homeostasis.

Members of the phospholipase Cγ (PLCγ) family link tyrosine kinase receptors to IP_3-mediated Ca^{2+} signals. We have previously shown that the PLCγ2 isoform is required for *in vitro* osteoclast development and *in vivo* bone homeostasis, however the mechanism how PLCγ2 regulates osteoclastogenesis has not yet been clearly identified. During osteoclast development, periodic changes in the intracellular calcium-levels – called calcium-oscillations – lead to the activation of NFATc1 master regulator transcription factor. The above issues prompted us to test the effect of PLCγ2 deficiency on the development of Ca^{2+}-level oscillations during osteoclastogenesis.

AIMS

In my PhD work I had the following objectives:

1) To test the role of PI3Kβ in *in vitro* osteoclast development and function.

2) To investigate the effect of the PI3Kβ$^{-/-}$ mutation on *in vivo* bone homeostasis under physiological conditions.

3) To identify the mechanism how PI3Kβ contributes to osteoclast development and function.

4) To test the role of PI3Kβ in ovariectomy-induced bone loss.

5) To identify the mechanism how PLCγ2 regulates osteoclast development.

METHODS

Animals. The *Pik3cb*$^{tm1.1Bvan/tm1.1Bvan}$ (referred to as PI3Kβ$^{-/-}$) mice carrying a homozygous deletion of exons 21-22 of the gene encoding for the p110β catalytic subunit of PI3Kβ were obtained from Dr. Bart Vanhaesebroeck. Heterozygous mice carrying a deleted allele of the PLCγ2-encoding gene (*Plcg2*^{tm1Jni}, referred to as PLCγ2^{-}) were provided by Dr. James N. Ihle. Transgenic mice ubiquitously expressing Lifeact-EGFP were obtained from Dr. Michael Sixt.

In vitro culture and resorption assays. Bone marrow cells were isolated from long bones (femur, tibia) of wild type, PI3Kβ$^{-/-}$ and PLCγ2$^{-/-}$ mice, and then differentiated into osteoclasts *in vitro* in the presence of recombinant M-CSF and RANKL. Osteoclast morphology was tested by tartrate-resistant acid phosphatase staining. For resorption assays, osteoclasts were cultured on an artificial hydroxyapatite layer or on bovine cortical bone slices.

For retroviral reconstitution of osteoclast precursors, fetal liver cells were obtained from wild type and PLCγ2$^{-/-}$ embryos, and then differentiated into osteoclasts *in vitro*.

Human osteoclasts were differentiated from peripheral blood mononuclear cells of healthy human volunteers obtained by dextran sedimentation and Ficoll-Paque gradient centrifugation. TRAP- staining and resorption assays were performed as described above.

Detection of apoptosis. For survival analysis, wild type and PI3Kβ$^{-/-}$ preosteoclasts were stained with Annexin-V-PE and 7-amino-actinomycin D, and analyzed on a flow cytometer. The number of apoptotic mature osteoclasts in the cultures was determined by the TUNEL-reaction.

Fluorescence microscopy. For F-actin staining, osteoclasts were fixed, permeabilized and stained with Alexa488-Phalloidin and DAPI. For live imaging of osteoclast development, myeloid precursors obtained from Lifeact-EGFP transgenic wild type and PI3Kβ$^{-/-}$ mice were cultured in the presence of M-CSF and RANKL, and imaged using a fluorescent microscope inside a tissue culture incubator. LysoTracker Red was used for acidic vesicle staining. For Ca^{2+} measurements the cells were loaded with Fura-2-AM and pluronic F127, and then imaged with a fluorescent microscope.

Analysis of gene expression. The measurement of osteoclast-specific gene expression was performed by quantitative PCR using Taqman assays.

Biochemical and signaling studies. Wild type and PI3Kβ$^{-/-}$ myeloid precursors were cultured in the presence of M-CSF and RANKL. The supernatants were collected and the proteins were precipitated with

acetone. Whole-cell lysates were obtained using a Triton X-100-based lysis buffer. Samples were run on SDS-page and immunoblotted.

Retroviral reconstitution. For the reexpression of PLCγ2, Platinum-E cells were transfected with a bicistronic murine stem cell virus-based retroviral vector expressing PLCγ2 along with GFP from an internal ribosome entry site. Viral supernatants were collected and incubated with fetal liver cells obtained from wild type and PLCγ2$^{-/-}$ embryos, and then the cells were differentiated into osteoclasts *in vitro*.

Micro-CT analysis. Trabecular bone structure and mineralization were tested by analysis of the distal metaphysis of the femurs of wild type and PI3Kβ$^{-/-}$ mice using a SkyScan 1172 micro-CT apparatus with NRecon and CT-Analyser softwares.

Histomorphometric analysis. Histomorphometry studies were performed on the distal metaphysis of the femurs of wild type and PI3Kβ$^{-/-}$ mice. Bones were fixed, decalcified, embedded in paraffine, sectioned and stained with TRAP, toluidine-blue and hematoxylin-eosin.

Ovariectomy. To test estrogen deficiency-induced bone loss, wild type and PI3Kβ$^{-/-}$ female mice were anesthetized and their ovaries were

surgically removed. 6 weeks after the operation, the mice were sacrificed, their femurs were excised and analyzed with micro-CT.

Statistical analysis. All experiments were performed three or more times - or on three or more individual mice - with comparable results. Statistical analysis was performed using Student's unpaired two-population t-test or two-way ANOVA with Tukey post-hoc comparison. For statistical analysis, Statistica 7.0 software was used. Level of significance was set at $p < 0.05$.

RESULTS

During my Ph.D. work, I analyzed the role of different intracellular signaling molecules in osteoclasts using transgenic mice. In the first part of the work, we investigated the role of PI3Kβ in *in vitro* osteoclast development and function as well as in *in vivo* bone homeostasis under physiological and pathological conditions.

When we tested the expression of the various PI3K isoforms during *in vitro* differentiation of mouse osteoclasts, we found that the expression of PI3Kβ - but not of the other PI3K isoforms - was dramatically upregulated. To assess the functional role of PI3Kβ in osteoclasts, we next tested the effect of the PI3Kβ inhibitor TGX221 on *in vitro* generated osteoclasts. Importantly, TGX221 dose-dependently inhibited development of osteoclasts from human or murine progenitors and their resorption capacity on an artificial hydroxyapatite surface.

To test the role of PI3Kβ in bone homeostasis, we turned to the analysis of the PI3Kβ$^{-/-}$ mice, and first evaluated the trabecular bone structure of the distal metaphysis of the femurs using micro-CT. Those experiments revealed significantly increased percent bone volume (BV/TV) in PI3Kβ$^{-/-}$ animals, which was primarily due to increased trabecular number rather than increased thickness of the individual trabeculae. We also performed histological and histomorphometric analysis on the femurs, and found that there was a moderate reduction

in the average number of osteoclasts in PI3Kβ$^{-/-}$ mice, while the PI3Kβ$^{-/-}$ osteoclasts had significantly shorter bone contact surface, and the depth of the resorption pits underneath the osteoclasts were dramatically reduced.

Next, we tested the effect of the PI3Kβ$^{-/-}$ mutation on *in vitro* osteoclast development and function. PI3Kβ deficiency led to reduced number of osteoclasts, as well as the PI3Kβ$^{-/-}$ cells showed defective resorptive capacity both on artificial hydroxyapatite surface and on bovine bone slices.

To better understand the role of PI3Kβ in osteoclasts, we next tested the expression of osteoclast-specific genes during osteoclastogenesis. The expression of genes encoding for TRAP, cathepsin K, integrin β_3-chain, NFATc1, calcitonin receptor and DC-STAMP was all strongly increased during osteoclast differentiation. PI3Kβ deficiency did not affect the expression of any of those genes, indicating that PI3Kβ is not required for osteoclast-specific gene expression. We also tested the survival of osteoclasts, and found that PI3Kβ deficiency does not affect survival or apoptosis of osteoclast-lineage cells. However, PI3Kβ$^{-/-}$ osteoclasts failed to form proper actin rings, which was further confirmed by the defective organization of the actin cytoskeleton in the Lifeact-EGFP expressing PI3Kβ$^{-/-}$ cells. In addition, PI3Kβ$^{-/-}$ osteoclasts showed a dramatic accumulation of intracellular acidic vesicles, and failed to release cathepsin K into the

extracellular space, suggesting defective discharge of cathepsin K-containing acidic vesicles.

Besides the role of PI3Kβ under physiological conditions, we also tested the effect of PI3Kβ deficiency on estrogen deficiency-induced bone loss. Though all mice lost trabecular bone following surgical ovariectomy, the relative amount of bone loss in PI3Kβ$^{-/-}$ mice was approximately half of that in their wild type counterparts, therefore the difference in trabecular bone volume (BV/TV) between the two genotypes became even more pronounced in the ovariectomized than the sham-operated animals.

In the second part of my Ph.D. work, I tested the role of PLCγ2 in the development of osteoclasts. We had previously shown that PLCγ2 is required for *in vitro* osteoclast development and function as well as for *in vivo* bone homeostasis. Our initial experiments showed clear TRAP-positive staining of PLCγ2$^{-/-}$ cultures, and our gene expression studies have not revealed any significant difference in the expression of osteoclast-specific genes between wild type and PLCγ2$^{-/-}$ cells.

Next we tested the role of PLCγ2 in the development of calcium-oscillations during osteoclastogenesis. In these experiments we found that the calcium-oscillations were present at 72 hours after RANKL induction in the wild type cultures. More importantly, the genetic deficiency of PLCγ2 completely blocked Ca^{2+}-oscillations in the PLCγ2$^{-/-}$ osteoclast cultures. Retroviral reintroduction of PLCγ2 into

PLCγ2$^{-/-}$ fetal liver derived osteoclast cultures restored the ability of the cells to show oscillations in the intracellular Ca^{2+}-levels.

Based on the results presented in my Ph.D. thesis, the PI3Kβ and PLCγ2 proteins may be suitable therapeutic targets in diseases characterized by excessive osteoclast-mediated bone resorption, such as osteoporosis and rheumatoid arthritis. Depending on the role of PI3Kβ in osteoclast development and osteoclast-mediated bone resorption, the isoform-specific PI3Kβ inhibitors may have novel therapeutic indications in bone diseases or may trigger unfavorable novel side effects by changing the balance of bone homeostasis.

CONCLUSIONS

The results of our studies lead us to the following conclusions:

1) PI3Kβ is required for *in vitro* osteoclast development and function.

2) PI3Kβ is required for *in vivo* bone homeostasis.

3) PI3Kβ is required for the regulation of actin ring formation, acidic vesicle discharge and release of cathepsin K in osteoclasts.

4) PI3Kβ is required for surgical ovariectomy-induced bone loss.

5) PLCγ2 is required for the development of calcium-oscillations during osteoclastogenesis.

PUBLICATIONS

Publications relevant to the dissertation:

1) **Győri D**, Csete D, Benkő S, Kulkarni S, Mandl P, Dobó-Nagy C, Vanhaesebroeck B, Stephens L, Hawkins PT, Mócsai A. (2014) The Phosphoinositide 3-Kinase Isoform PI3Kβ Regulates Osteoclast-Mediated Bone Resorption in Humans and Mice. *Arthritis & Rheumatology*, 66(8):2210–2221.
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2) Kertész Z, **Győri D**, Körmendi S, Fekete T, Kis-Tóth K, Jakus Z, Schett G, Rajnavölgyi É, Dobó-Nagy C, Mócsai A. (2012) Phospholipase Cγ2 is required for basal but not oestrogen deficiency-induced bone resorption. *European Journal of Clinical Investigation*, 42:49–60.
IF: 3.365

Other publications:

3) Boyle KB, **Győri D**, Sindrilaru A, Scharffetter-Kochanek K, Taylor PR, Mócsai A, Stephens LR, Hawkins PT. (2011) Class IA Phosphoinositide 3-Kinase β and δ Regulate Neutrophil Oxidase Activation in Response to Aspergillus fumigatus Hyphae. *The Journal of Immunlogy*, 186:2978–2989.
IF: 5.788