The role of PLCγ2 and p190RhoGAP in osteoclast differentation and function

Ph.D. thesis

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Budapest
2011

1 Introduction

The skeleton is the body part that forms the supporting structure of the organism. It is composed of mineralized tissue and is typical of many vertebrates. The skeleton often serves as an attachment site for muscles and a mechanism for transmitting muscular forces. It also has an important function as a reservoir for calcium and phosphorus. To fulfill the structural function, not only the amount of bone tissue present in the skeleton is important, but also the architecture and shape of bones. Bone tissue is not static, and healthy bones require continuous remodeling and modeling to adapt to their dual roles as a supporting frame and as a regulator of mineral homeostasis. Remodeling is a lifelong process where mature bone tissue is removed from the skeleton (a process called bone resorption) and new bone tissue is formed (a process called ossification or new bone formation). If the two processes are quantitatively equal, the remodeling is balanced. An imbalance in the regulation of bone remodeling's two sub-processes results in many metabolic bone diseases, such as osteoporosis. Osteoporosis is characterized by low bone mass and deterioration of bone structure, which results in bone fragility and increased risk of fracture.

The cells responsible for bone resorption are the osteoclasts. They are derived from the hematopoietic lineage and differentiate from macrophage precursors. Osteoclasts are formed by the fusion of mononuclear precursors into huge multinucleated cells. The maintenance of the balance in the skeletal system requires another process, which is the bone formation. The cells responsible for new bone secretion are the osteoblasts. They are derived from mesenchymal stem cells and osteoblasts play a critical role in regulation of osteoclast function.

The phospholipase Cγ2 (PLCγ2) enzyme is expressed in hematopoietic lineage-derived cells. The role of PLCγ2 has been previously described in B-cells, platelets and other cell types of the immune system, like NK cells, mast cells, macrophages. PLCγ2 is activated by Fc receptors and integrins. The lack of PLCγ2 causes impaired immunreceptor-like signaling in neutrophil granulocytes.

The p190RhoGAP protein has two isotypes: p190-A and p190-B. The p190RhoGAPs are involved in integrin signaling pathway in several hematopoietic cell lines, and it is well known that the β3 integrins have essential role in osteoclast differentation and function. Deficiency of p190-A or p190-B causes embryonic lethality in mice.

2 Objectives

2.1 What is the role of PLCγ2 in osteoclast differentation and function?

2.2 In which signaling pathway can PLCγ2 be involved in osteoclasts?

2.3 Does PLCγ2 have any role in *in vivo* bone metabolism under normal and estrogen-deficient conditions?

2.4 How can we overcome the embryonic lethality of p190RhoGAP$^{-/-}$ animals and investigate the function of p190-A and p190-B in osteoclasts?

2.5 Does p190RhoGAP have any role in osteoclasts differentation and function?

3 Materials and methods

3.1 Animals

Heterozygous mice carrying a deleted allele of the PLCγ2-encoding gene (*Plcg2*^{tm1Jni}, referred to as PLCγ2^{-}) were obtained from James N. Ihle (St. Jude Children's Research Hospital, Memphis, TN, USA). Mice carrying deleted allele of p190-A or p190-B–encoding gene were generated by Dr. Jeffrey Settleman (Massachussets General Hospital, Boston, MA, USA). Due to the embryonic lethality of homozygous p190RhoGAP$^{-/-}$ mice, the *in vitro* experiments were performed on cells from p190RhoGAP$^{-/-}$ (and appropriate control) bone marrow chimeras.

3.2 *In vitro* cultures and resorption assays

For *in vitro* bone marrow cell cultures, the cells were isolated from bone marrow of the femurs and tibias. Suspensions of bone marrow cells were cultured for 48 h in α-MEM (Invitrogen) in the presence of 10 ng/ml recombinant mouse M-CSF (Peprotech). Cellular morphology and tartrate resistant acid phosphatase (TRAP) expression was determined after 3-5 days culture in 24-well tissue culture-treated plates using a commercial TRAP staining kit (Sigma). For *in vitro* resorption

assays, osteoclast precursors were plated on BioCoat Osteologic slides (BD Biosciences), cultured in the presence of M-CSF and RANKL for 10-14 days and processed according to the manufacturer's instructions. Cultures were observed and imaged using a Leica Microsystems (Wetzlar) DMI6000B inverted microscope. The number of osteoclasts (i. e. TRAP positive cells with 3 or more nuclei) was counted manually, while the percentage of resorbed area was determined using the ImageJ software.

3.3 Analysis of gene expression

Osteoclast-specific gene expression was tested using quantitative real-time PCR analysis from wild type or PLC$\gamma 2^{-/-}$ cultures generated in the indicated periods of time using the indicated cytokine concentrations. Total RNA was then isolated from the cells with Trizol reagent (Invitrogen). Reverse transcription was performed at 37 °C for 120 minutes from 100 ng total RNA using the High Capacity cDNA Archive Kit (Applied Biosystems). Quantitative real-time PCR reactions were performed in triplicates with a control reaction containing no reverse transcriptase on an ABI PRISM 7900 (Applied Biosystems) equipment with 40 cycles at 94 °C for 12 seconds and 60 °C for 60 seconds using Applied Biosystems

Taqman Gene Expression Assay kits. We tested the expression of the mouse *Acp5* (TRAP), *Calcr* (Calcitonin receptor), *Ctsk* (cathepsin K), *Fos* (c-Fos), *Nfatc1* (NFATc1), *Oscar* (OSCAR) and *Tm7sf4* (DC-STAMP) genes and normalized those to the expression of the housekeeping gene *Gapdh* (GAPDH). The comparative C_t method was used to quantify transcripts.

3.4 Biochemical and signaling studies

For the detection of PLCγ2, p190-A and p190-B protein expression, whole cell lysates were isolated from osteoclast/macrophage or embryonic brain samples using Triton-X lysis buffer supplemented with protease and phosphatase inhibitors.

For signaling studies, macrophages were cultured in bacterial dishes for 5-8 days in the presence of M-CSF supplied in the form of a 10% conditioned medium from CMG14-12 cells, then suspended with 5 mM EDTA and serum starved for 6 h. When indicated, the cells were incubated with 10 μM PP2 (EMD Biosciences) for 8 min. The cells were stimulated with 50 ng/ml M-CSF or 50 ng/ml RANKL in suspension or were plated on 6-cm tissue culture-treated dishes. The reaction was stopped after 30 min at 37 °C and whole cell lysates were prepared. PLCγ2 was precipitated using the Q-20 PLCγ2

antibody (Santa Cruz Biotechnology) and captured using a 1:1 mixture of Protein A Sepharose (Zymed) and Protein G Agarose (Invitrogen). Whole-cell lysates or PLCγ2 immunoprecipitates were immunoblotted with phosphorylation-specific antibodies (from Cell Signaling Technology) against PLCγ2 (pTyr 759; #3874), ERK (#9101) or the p38 MAP kinase (#9211); non-phospho-specific antibodies against PLCγ2 (Q-20; Santa Cruz), ERK1/2 (combination of C-16 (ERK1) and C-14 (ERK2) from Santa Cruz), p38 MAP kinase (C-20; Santa Cruz), IκBα (#9242; Cell Signaling) or β-actin (AC-74; Sigma); or antibodies against phosphotyrosine (clone 4G10; Millipore). Signal intensity was developed using secondary antibodies and ECL reagents from GE Healthcare.

3.5 Ovariectomy

To test estrogen deficiency-induced bone loss, wild type and PLCγ2$^{-/-}$ females at 8 weeks of age were anesthetized with ketamine and medetomidine and subjected to surgical ovariectomy or sham operation. 6 weeks after the operation, the mice were sacrificed and their femurs or tibias were analyzed.

3.6 Micro-CT and histomorphometry

Bone architecture under basal conditions was tested on age-matched wild type and PLCγ2$^{-/-}$ male mice at 8-10 weeks of age. Ovariectomy-induced bone loss was tested at 14 weeks of age on wild type and PLCγ2$^{-/-}$ females.

Micro-CT studies were performed on the distal metaphysis of the femurs stored in PBS containing 0.1% Na-azide. Samples were scanned on a SkyScan 1172 micro-CT apparatus using a 50 kV and 200 μA X-ray source with 0.5 mm aluminium filter, and a rotation step of 0.5° with frame averaging turned on, resulting in an isometric voxel size of 4.5 μm. Three-dimensional images were reconstituted and analyzed using the NRecon and CT-Analyser software (both from SkyScan). For quantitative analysis, 400 horizontal sections starting 50 sections above the distal growth plate were selected, and the boundaries of trabecular area were selected manually a few voxels away from the endocortical surface. The density threshold for bone tissue was set manually by an experienced investigator. For graphical presentation, the two-dimensional representation of a horizontal section 250 sections above the distal growth plate, as well as the three-dimensional reconstitution of an axial cylinder of 700 μm diameter,

expanding from 150 to 450 sections above the distal growth plate have been prepared.

Histomorphometry studies were performed on the proximal metaphysis of the tibias. After sacrificing the mice, the bones were placed in 70% ethanol, then fixed overnight in 4% formalin and embedded undecalcified in methylmetacrylate (Technovit). After polymerization, 3-4 μm sections were cut with a Jung micrometer and deplastinated in methoxymethylmetacrylate (Merck). Sections were stained with von Kossa and Goldner stains. Bone histomorphometry was performed using a microscope (Nikon) equipped with a video camera and digital analysis system (OsteoMeasure, OsteoMetrics). Histomorphometry parameters were measured according to international standards.

3.7 Statistical analysis

Experiments were performed the indicated times with comparable results. Statistical analyses were performed using Student's unpaired two-population t-test with unequal variance. Analysis of the interaction between the effects of genotypes and surgical treatments was performed by two-way ANOVA. p values below 0.05 were considered statistically significant.

4 Results

4.1 Investigation of the role of PLCγ2

4.1.1 PLCγ2 is required for *in vitro* osteoclast development

To test the role of PLCγ2 in osteoclasts, we have cultured wild type and PLCγ2$^{-/-}$ bone marrow cells *in vitro* under osteoclastogenic conditions. The concentration of M-CSF and RANKL were changed between 20ng/ml and 50ng/ml. In wild type cultures we were able to detect huge, multinuclear osteoclasts staining positive for TRAP. PLCγ2$^{-/-}$ cultures consistently stained positive for TRAP, but we couldn't detect any osteoclasts even at the highest cytokine concentrations.

Taken together, PLCγ2 is required for the *in vitro* development of mature multinucleated osteoclasts in the presence of M-CSF and RANKL but likely not for the initial steps of preosteoclast differentiation.

We next tested the effect of PLCγ2 deficiency on osteoclast-mediated bone resorption by culturing bone marrow cells on an artificial hydroxiapatite layer. Wild type cells cultured in the presence of 20 ng/ml M-CSF and 20 ng/ml RANKL had a moderate resorptive capacity which was strongly increased by increasing the concentration of both cytokines to 50 ng/ml. In

contrast, practically no resorption could be observed in PLCγ2$^{-/-}$ cultures at either cytokine concentrations. Therefore, PLCγ2 is also required for osteoclast-mediated bone resorption, likely reflecting the previously mentioned osteoclast developmental defect.

4.1.2 PLCγ2 is not required for expression of osteoclast-specific genes

Our next aim was to address whether PLCγ2 is involved in an earlier or a later phase of osteoclast differentiation. Since we were able to obtain normal numbers of apparently normal macrophages from PLCγ2$^{-/-}$ bone marrow cells and those macrophages expressed normal levels of the macrophage differentiation marker F4/80, it is unlikely that PLCγ2 is required for the first steps of general myeloid cell differentiation.

We next tested the time course of osteoclast-specific gene expression in *in vitro* cultures by quantitative RT-PCR. The expression of the *Acp5* (encoding for TRAP), *Calcr* (calcitonin receptor), *Ctsk* (cathepsin K), *Fos* (c-Fos), *Nfatc1* (NFATc1), *Oscar* (OSCAR) and *Tm7sf4* (DC-STAMP) genes was strongly increased during osteoclast differentiation in wild type cultures but none of those genes showed increased expression in

parallel macrophage samples. The genetic deficiency of PLCγ2 did not induce any major reduction of osteoclast-specific gene expression, though some partial decrease could be observed, particularly in the case of the expression of *Calcr*. Most importantly, the expression of the genes encoding for the early maturation marker TRAP (*Acp5*), the most plausible PLCγ2 effector NFATc1 (*Nfatc1*) and DC-STAMP (*Tm7sf4*), a critical player of the preosteoclast fusion machinery were all upregulated normally in PLCγ2$^{-/-}$ cultures. These results indicate that PLCγ2 is mostly dispensable for initiation of osteoclast-specific gene expression.

4.1.3 Biochemical characterization of the PLCγ2-mediated osteoclast signaling pathway

Osteoclast development is triggered by three major extracellular signals: M-CSF, RANKL and adhesive interactions with the environment (e. g. with tissue culture plastic surface). We next tested which of those three signals trigger PLCγ2 activation, using wild type macrophages stimulated with M-CSF or RANKL in suspension (which was required to avoid parallel engagement of adhesion receptors), or plated on a tissue culture plastic surface. PLCγ2 phosphorylation occured upon adhesion of macrophages but

not upon M-CSF or RANKL stimulation in suspension. However, M-CSF-induced ERK phosphorylation and RANKL-induced p38 MAP kinase phosphorylation and NFκB activation (degradation of IκBα) could readily be observed under those conditions, indicating intact basic M-CSF and RANKL signaling in suspension. Therefore, PLCγ2 appears to be activated by adhesive interactions rather than by stimulation with M-CSF or RANKL cytokines.

We have also tested the role of Src-family kinases in PLCγ2 phosphorylation. Pretreatment of macrophages with the Src-family inhibitor PP2 completely abrogated the PLCγ2 phosphorylation response, indicating that the adhesion-induced PLCγ2 activation requires members of the Src kinase family.

4.1.4 micro-CT and histomorphometric analysis of wild type and PLCγ2–/– animals

Our previous results verified that PLCγ2 has important role during osteoclast differentation and function, but its role in *in vivo* bone homeostasis was poorly understood. So we next analyzed the composition of trabecular bone of wild type and PLCγ2$^{-/-}$ male mice using micro-CT analysis of the distal metaphysis of the femurs. The number of the trabeculas in

PLCγ2$^{-/-}$ animals were significantly increased compared to the wild type animals. Quantification of the entire three-dimensional reconstitution image revealed a significant increase in the percent bone volume (BV/TV) of PLCγ2$^{-/-}$ mice (p=0.011; n=5). Histomorphometric analysis on the trabecular bone of the proximal tibias of wild type and PLCγ2$^{-/-}$ animals were also performed with the same results. Taken together, PLCγ2$^{-/-}$ mice have increased trabecular bone volume likely due to a defect in osteoclast development and function.

Next, we tested the role of PLCγ2 in pathological bone loss by subjecting wild type and PLCγ2$^{-/-}$ animals to estrogen deficiency-induced bone loss. As expected, surgical ovariectomy led to a significant reduction in the per cent bone volume (BV/TV) of wild-type mice (p=0.025; n=7). Contrary to our expectations, the per cent bone volume of PLCγ2$^{-/-}$ animals was also significantly reduced (p=0.00023; n=4) and that reduction was even higher in PLCγ2$^{-/-}$ mice than in wild-type animals both in terms of absolute reduction in BV/TV values (4.1 vs. 1.6 percentage points, respectively) and in percentage of the BV/TV values of the sham-operated control animals (50% vs. 36%, respectively). The difference of the effect of ovariectomy on wild-type and PLCγ2$^{-/-}$ animals

(interaction of the genotypes and surgical procedures) proved to be statistically significant (p=0.0090). Importantly, while the BV/TV values of sham-operated wild-type and PLCγ2$^{-/-}$ animals were statistically highly significant (p=0.00025), there was no significant difference between the two genotypes after the ovariectomy procedure (p=0.25; n=7 (wild type) vs. n=4 (PLCγ2$^{-/-}$)). Similar differences could be observed in the trabecular numbers, whereas the trabecular thickness remained unaffected by the different genotypes and surgical procedures.

The above-mentioned findings were also confirmed by histomorphometric analysis of ovariectomy-induced bone loss in the proximal tibia. Additional studies testing the number of osteoclasts and osteoblasts indicated that although the number of osteoclasts was significantly lower in the sham-operated PLCγ2$^{-/-}$ mouse than in the wild type one, the number of osteoclasts was strongly increased and reached a comparable level in the two genotypes after the ovariectomy procedure.

Taken together, these results suggest that ovariectomized PLCγ2$^{-/-}$ animals are capable of reducing their bone mass to levels comparable to those seen in similarly treated wild type animals, possibly because of a comparable estrogen deficiency-induced increase in osteoclast numbers in the two genotypes.

4.2 Role of p190RhoGAPs in osteoclast differentation and function

4.2.1 Generation of p190-A or B deficient osteoclast cultures *in vitro*

The perinatal lethality of p190RhoGAP$^{-/-}$ mice precluded the analysis of p190RhoGAP$^{-/-}$ osteoclasts from adult mice. To overcome this, chimeric mice with a p190RhoGAP$^{-/-}$ hematopoietic compartment were generated. To this end, lethally irradiated recipients carrying the CD45.1 allele were injected i.v. with fetal liver cells of p190RhoGAP$^{-/-}$ or wildtype control fetuses (both donor strains carry the CD45.2 allele). Flow cytometric analysis of peripheral blood samples 4–6 wk after transplantation revealed that routinely more than 98% of the circulating neutrophils of wild-type and p190RhoGAP$^{-/-}$ chimeras, respectively, were of donor origin (CD45.2 positive). Immunoblot analyses confirmed that bone marrow-derived macrophages isolated from p190RhoGAP$^{-/-}$ chimeras did indeed lack the p190RhoGAP proteins (both p190-A and p190-B).

4.2.2. Role of p190-A and p190-B in osteoclasts differentation and function

After estabilishing the bone marrow transplantation procedure of p190RhoGAP$^{-/-}$ animals, we were able to generate p190-A and p190-B deficient bone-marrow cell cultures to investigate the role of p190RhoGAPs in osteoclasts differentation and function.

When analizing p190-A cell cultures we found that both wild type and p190-A$^{-/-}$ cells were able to form huge, multinuclear TRAP positive cells and we could not observe any difference in the number of osteoclast-like cells. The p190-A deficient osteoclasts were able to digest the artificial hydroxyapatite surface in a comparable level to the wild type cells.

The osteoclast cultures of wild type and p190-B$^{-/-}$ chimeras showed similar capability to form huge, multinucleated TRAP positive cells. The p190-B$^{-/-}$ osteoclasts exhibited a similiar or more pronounced resorption on artificial hydroxyapatite surface than the wild type cells.

Taken together, these results suggest that neither p190-A nor p190-B has an important role during *in vitro* osteoclasts differentation and function.

5 Discussion

We investigated the role of PLCγ2, p190-A and p190-B proteins in *in vitro* osteoclasts differentation and function and - in the case of PLCγ2 - its function in *in vivo* bone homeostasis under normal and pathological conditions.

Our results indicate that:

5.1. PLCγ2 is required for *in vitro* osteoclasts differentation and function.

5.2. PLCγ2 is indispensable for upregulation of osteoclasts specific gene expression.

5.3. PLCγ2 is activated by an adhesion-dependent manner.

5.4. PLCγ2 is required for basal bone homeostasis.

5.5. PLCγ2 is not required for estrogen deficiency-induced bone loss.

5.6. Basal and estrogen deficiency-induced bone loss uses different signaling pathways.

5.7. Embryonic liver cells are able to maintain bone marrow cells and form osteoclasts after bone-marrow transplantation.

5.8. The p190-A and p190-B proteins are indispensable for *in vitro* osteoclasts differentation and function.

6 Publication list

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IF: 2,643

6.2. Tamás Németh, Krisztina Futosi, Csilla Hably, Madelaine R. Brouns, Sascha M. Jakob, Miklós Kovács, **Zsuzsanna Kertész,** Barbara Walzog, Jeffrey Settleman and Attila Mócsai: *Neutrophil functions and autoimmune arthritis in the absence of p190RhoGAP: generation and analysis of a novel null mutation in mice.*

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