The role of Syk and PI3Kβ proteins in osteoclast development, function and bone homeostasis

Ph.D. Theses

Dániel Csete M.D.

Semmelweis University

Ph.D. School of Molecular Medical Sciences

Supervisor: Attila Mócsai, M.D., Ph.D., DSc

Reviewers: Zsolt Rónai, M.D., Ph.D.
Virág Vas, Ph.D.

Chairman of the final examination committee:
Zsuzsanna Fürst, M.D., Ph.D., DSc

Members of the final examination committee:
Szabolcs Sipeki, M.D., Ph.D.
Gergely Szakács, Ph.D.

Budapest

2019

Introduction

Osteoclasts are unique, bone resorbing, multinuclear cells of hematopoietic origin formed by biochemical maturation and fusion of myeloid precursors. The first stage of osteoclast development is similar to macrophage development: the hematopoietic stem cell differentiates into myeloid precursor, then macrophage-osteoclast-precursor in the presence of M-CSF (macrophage colony stimulating factor). These precursors further differentiate into osteoclasts in response to RANK ligand (receptor activator of NFκB ligand) produced by osteoblasts and integrin-mediated activation.

The immunoreceptor-like signaling pathways required for osteoclast development activate ITAM containing adapter proteins which leads to Syk activation. We have *in vitro* data about the severe damage in osteoclast development in the absence of Syk. Because of the embryonal lethality of $Syk^{-/-}$ mice no *in vivo* data is available about the role of Syk in bone homeostasis. Therefore, lineage-specific Syk deletion is required for such investigations.

The role of Syk in osteoclast was confirmed in $\alpha_v\beta_3$ integrin mediated signaling too. Syk activation is required for cytoskeletal rearrangement which is crucial for osteoclast function. The inhibition of Syk might be protective in models characterized by increased bone loss such as rheumatoid arthritis or collagen induced arthritis. Based on these data it is important to clarify the role of Syk in *in vivo* bone homeostasis.

It is known that the phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin blocks osteoclast development, but the specific role of the different isotypes was unknown. Our workgroup has described the importance of PI3Kβ in the osteoclast development and function. To clarify the mechanism of this effect, we analyzed different osteoclast-specific functions in PI3Kβ KO mice, and found the actin ring development to be crucial. In the absence of PI3Kβ not only the number of osteoclasts was reduced, but also the ratio of actin ring formation was strongly decreased and there was practically no actin ring development in the PI3Kβ KO osteoclasts. Analyzing the dynamic changes of actin rings in mature osteoclast after PI3Kβ-specific inhibitors was one of our next objectives.

Objectives

Our objectives were to answer the following questions:

1) What is the effect of the lineage-specific Syk deletion on the *in vivo* bone homeostasis?
2) What is the effect of the lineage-specific Syk deletion on the *in vitro* osteoclast development and function?
3) What causes the differences between the effects of osteoclast specific and hematopoietic deletion?
4) What is the effect of the isoform-specific inhibition of PI3Kβ on the actin ring maintenance of osteoclasts?

Methods

Animals

We used mice with C57BL/6 genetic background. All animal experiments were approved by the Animal Experimentation Review Board of the Semmelweis University.

Syk-deficient mice

Mice carrying the *Syk*$^{tm1.2Tara}$ (referred to as *Syk*^{flox}) floxed allele of the *Syk* gene were obtained from Alexander Tarakhovsky (Rockefeller University) and were maintained in homozygous form (*Syk*$^{flox/flox}$).

Mice carrying the *Ctsk*$^{tm1(cre)Ska}$ (referred to as *Ctsk*^{Cre}) knock-in mutation resulting in the osteoclast-specific expression of the Cre recombinase under the control of the endogenous promoter of the *Ctsk* gene and at the same time inactivating the Ctsk gene were obtained from Shigeaki Kato (University of Tokyo) and were maintained in heterozygous form (referred to as Ctsk-Cre).

Mice carrying the *Commd10*$^{Tg(Vav1-icre)A2Kio}$ transgenic insertional mutation expressing the Cre recombinase in the entire haemopoietic lineage from the exogenous Vav1 promoter and at the same time inactivating the Commd10 gene were obtained from the Jackson Laboratory and were maintained in heterozygous form (referred to as Vav-Cre).

Osteoclast-specific deletion of Syk was achieved by crossing the Ctsk-Cre and $Syk^{flox/flox}$ mice to obtain $Ctsk^{Cre/+}Syk^{flox/flox}$ (referred to as $Syk^{\Delta OC}$) animals. Deletion of Syk in the entire hematopoietic compartment was achieved by crossing the Vav-Cre and $Syk^{flox/flox}$ mice to obtain $Commd10^{Tg(Vav1\text{-}icre)A2Kio/+}Syk^{flox/flox}$ (referred to as $Syk^{\Delta Haemo}$) animals.
The allele obtained by Cre-mediated deletion of the Syk^{flox} allele will be referred to as the Syk^{Δ} allele.

PI3Kβ-deficient mice

Mice carrying the $Pik3cb^{tm1.1Bvan/tm1.1Bvan}$ mutation (referred to as PI3Kβ$^{-/-}$) were obtained from Bart Vanhaesebroeck (Barts Cancer Institute, Queen Mary University of London) and were maintained by crossing PI3Kβ$^{-/-}$ female mice and PI3Kβ$^{+/-}$ male mice because of the infertility of a PI3Kβ$^{-/-}$ male mice. In these mice exon 21 and 22 are deleted from the catalytic subunit of PI3Kβ.

A Lifeact-EGFP system

Transgenic mice ubiquitously expressing Lifeact-EGFP were obtained from Dr. Michael Sixt (Institute of Science and Technology, Klosterneuburg, Austria).

Micro-CT analysis

Mice were sacrificed at 9 weeks of age and their right femurs were subjected to micro-CT analysis by a SkyScan 1172 microCT apparatus. For quantitative analysis relative bone volume (BV/TV), trabecular number, trabecular thickness, trabecular separation and SMI (structure model index) were calculated.

Histological procedures and immunostaining

Femurs isolated from mice at 9 weeks of age were fixed in 4% paraformaldehyde (Sigma-Aldrich) followed by decalcification in Osteomoll (Merck) for 3 weeks. For immunostaining of the calcitonin receptor, we used anti-Calcitonin Receptor (Abcam AB11042) and anti-rabbit Alexa Fluor 488 (Life Technologies, A11034) antibodies. Microscopic images were taken by a Nikon ECLIPSE Ni-U microscope connected to a Nikon DS-Ri2 camera.

In vitro culture and resorption assays

Bone marrow derived cells from wild type, $Syk^{\Delta OC}$ and $Syk^{\Delta Haemo}$ mice were obtained from femur and tibia by flushing with PBS, and cultured in α-MEM medium (Sigma) (supplemented by 10% FCS (Gibco), 1% LGlutamin and 1% antibiotics) in the presence of 10 ng/ml recombinant MCSF (Peprotech) for 2 days. Non-adherent cells were plated on tissue culture treated surface in the concentration of 1.5×10^5 cell/cm^2 and differentiated into osteoclasts in the presence of 50 ng/ml M-CSF and 50

ng/ml RANKL (Peprotech). Under identical condition, but without RANKL treatment, cells were differentiated into macrophages.

Cultures were terminated and osteoclast-specific staining was performed using a commercial tartrate-resistant acid phosphatase (TRAP) staining kit (Sigma-Aldrich).

For *in vitro* resorption assay cells were cultured under identical conditions except the 7 days long 50 ng/ml M-CSF and 50 ng/ml RANKL treatment and the hydroxyapatite surface (Sigma-Aldrich) followed by washing (10 % Hypochlorous acid).

Biochemical studies

For protein content analysis osteoclast or macrophage cultures were terminated, whole cell lysates were run on SDS-page, then immunoblotted.

Quantitative RT-PCR analysis

Osteoclast-specific gene expression was analyzed by quantitative real-time PCR. Osteoclast or macrophage cultures were terminated and after RNA preparation (TriPure, Roche) osteoclast-specific Taqman assays were used.

Genomic PCR analysis

Osteoclast or macrophage cultures were washed, followed by isolation of genomic DNA and PCR using standard procedures.

Actin ring analysis

After 3 days of RANKL treatment the actin ring structures were formed by mature osteoclasts. Cultures were treated by 50 nM PI3Kβ-specific inhibitor (TGX221) or vehicle (DMSO). Microscopic images were taken by Nikon BioStation IM-Q.

Statistical analysis

Experiments were performed three or more times. Micro-CT measurements were analyzed by two-way (factorial) ANOVA. Other measurements were analyzed by one-way ANOVA followed by Tukey or Unequal N HSD post hoc test. We used Statistica 8.0 software for statistical analysis, p values below 0.05 were considered statistically significant.

Results

The role of Syk in osteoclast development and bone homeostasis

Micro-CT – osteoclast-specific Syk deletion

Because of the perinatal lethality of *Syk*$^{-/-}$ mice analyzing the bone morphology of adult *Syk*$^{-/-}$ mice was impossible. To overcome that problem, we generated lineage-specific Syk-deficient animals which were analyzed by micro-CT.

As shown in the longitudinal sections of the femurs of *Syk*$^{\Delta OC}$ mice and the appropriate controls the *Syk*$^{\Delta OC}$ mutation strongly increased the density of the trabecular area compared to wild type mice, whereas no dramatic difference could be observed in Ctsk-Cre or *Syk*$^{flox/flox}$ animals. Analysis of representative cross-sections and 3D reconstitution images of the femurs also showed similar tendencies.

According to the quantitative analysis of the micro-CT images the percent bone volume (BV/TV) was strongly increased in *Syk*$^{\Delta OC}$ mice, whereas no substantial difference could be observed in Ctsk-Cre or *Syk*$^{flox/flox}$ mice.

We have also performed statistical analysis by two-way (factorial) ANOVA which determines the interaction of the two (Ctsk-Cre and *Syk*$^{flox/flox}$) mutations. The presence of the two mutation in the *Syk*$^{\Delta OC}$ resulted in a statistically significant difference.

We observed remarkable differences in the trabecular number and trabecular separation, while no consistent change was observed in the trabecular thickness and SMI (structural model index) of the same mice. Taken together, our results indicate that osteoclast-specific deletion of Syk causes increased bone trabecular mass primarily due to increased bone trabecular number rather than a higher trabecular thickness.

Micro-CT – hematopoietic Syk deletion

Mice with Syk deficiency in the entire hematopoietic compartment (and their controls) were also analyzed by micro-CT. The longitudinal, cross-sectional and 3D reconstitution images showed robust increase in the bone density in case of the $Syk^{\Delta Haemo}$ mice, while no substantial changes were observed in Vav-Cre or $Syk^{flox/flox}$ animals.

Further quantitative analysis of the micro-CT data indicated a strongly increased percent bone volume (BV/TV) in $Syk^{\Delta Haemo}$ mice. Importantly, BV/TV values in $Syk^{\Delta Haemo}$ mice appeared to be substantially higher than corresponding $Syk^{\Delta OC}$ animals.

Similar to the $Syk^{\Delta OC}$ mice, the increased trabecular bone volume was primarily due to an increased trabecular number rather than trabecular thickness. Trabecular separation was also reduced in $Syk^{\Delta Haemo}$ mice and the SMI value changed significantly.

Taken together, early deletion of Syk in the entire hematopoietic system resulted in dramatic increase in the mineralized trabecular bone mass, indicating a critical role for Syk in *in vivo* bone homeostasis.

There was a substantially lower BV/TV value in *Syk*$^{\Delta OC}$ mice compared to *Syk*$^{\Delta Haemo}$ animals, raising the possibility that the lower values in the *Syk*$^{\Delta OC}$ mutants might be due to incomplete deletion of Syk by Cre expression.

Bone histological analysis

We performed histological analysis of the distal femur of wild type, *Syk*$^{\Delta OC}$ or *Syk*$^{\Delta Haemo}$ mice. Compared to wild type animals, much denser trabecular network was seen in *Syk*$^{\Delta OC}$ and especially, *Syk*$^{\Delta Haemo}$ mice.
To test the presence of mature osteoclasts on the trabecular bone surface, we performed immunofluorescence staining of bone sections for calcitonin receptor, an osteoclast-specific differentiation marker. Calcitonin receptor signals were evident on the lining of trabecular rods in wild type sections. Similar signals were also seen but at substantially lower numbers in *Syk*$^{\Delta OC}$ sections, whereas no such signals were seen in *Syk*$^{\Delta Haemo}$ sections.

***In vitro* osteoclast development in lineage-specific Syk mutants**

We tested *in vitro* development of osteoclasts from wild type, *Syk*$^{\Delta OC}$ or *Syk*$^{\Delta Haemo}$ bone marrow cells in the presence of recombinant M-CSF and RANKL cytokines. TRAP-staining was performed at certain time points during osteoclastogenesis to observe osteoclast morphology.
No TRAP-positive multinuclear cells (osteoclasts) were seen 2 days after addition of RANKL to the cultures. However, osteoclasts started to appear in wild type cultures on day 3 and formed very large

multinucleated TRAP-positive cells 3.5 days after the initial RANKL treatment. Some osteoclasts also formed in *Syk*$^{\Delta OC}$ cultures, though they were much smaller in size, and practically no osteoclasts) could be observed in *Syk*$^{\Delta Haemo}$ cultures.

The above results confirm prior studies indicating a critical role for Syk during *in vitro* osteoclast development. They also indicate an incomplete osteoclast developmental defect in *Syk*$^{\Delta OC}$ cultures (as opposed to the complete defect in *Syk*$^{\Delta Haemo}$ ones), suggesting incomplete deletion of Syk in *Syk*$^{\Delta OC}$ mutants.

Analysis of the *in vitro* resorptive activity of osteoclasts

We also attempted to test the *in vitro* resorbing capacity of osteoclasts by an assay measures the combined effect of both osteoclast development and osteoclast-mediated matrix resorption. To this end, myeloid precursors were plated on an artificial hydroxyapatite layer and cultured for 7 days.

Wild type osteoclast cultures were able to resorb substantial areas of the hydroxyapatite layer. In contrast, only small areas of resorption could be observed in *Syk*$^{\Delta OC}$ cultures and no resorption was seen in *Syk*$^{\Delta Haemo}$ cultures.

Analysis of osteoclast-specific gene expression

We tested the changes of osteoclast-specific gene expression in osteoclast cultures from the different genotypes. The expression of DC-STAMP (encoded by the *Tm7sf4* gene), TRAP (*Acp5*), calcitonin receptor (*Calcr*),

NFATc1 (*Nfatc1*) and cathepsin K (*Ctsk*) mRNA strongly increased upon osteoclastic differentiation whereas no such increase could be observed in parallel macrophage cultures. The expression of all those genes were reduced in both the $Syk^{\Delta OC}$ and $Syk^{\Delta Haemo}$ cultures. Gene expression data indicate a role for Syk in regulation of osteoclast-specific gene expression.

Analysis of Syk protein levels

The different severity of the *in vivo* bone phenotypes and *in vitro* osteoclast developmental defect between the $Syk^{\Delta OC}$ and $Syk^{\Delta Haemo}$ mutants raised the possibility that Syk is incompletely deleted from $Syk^{\Delta OC}$ osteoclasts. To test this more specifically, we performed Western Blot analysis of Syk expression during osteoclast development.

Syk was present in wild type cultures and its expression slightly even increased during osteoclast differentiation. Syk was also present throughout the assessment period in $Syk^{\Delta OC}$ cultures, but was completely absent throughout the entire observation period in $Syk^{\Delta Haemo}$ cultures.

Semiquantitative analysis confirmed the presence of Syk in all wild type and $Syk^{\Delta OC}$ but not in $Syk^{\Delta Haemo}$ samples. Although there was a tendency of reduced Syk expression in $Syk^{\Delta OC}$ osteoclasts as compared to wild type osteoclasts, this difference was not statistically significant, indicating that the $Syk^{\Delta OC}$ mutation is not able to reduce Syk expression at the overall cell population level.

The above results provided direct evidence supporting our assumption that Syk is incompletely deleted from $Syk^{\Delta OC}$ but it is completely absent from $Syk^{\Delta Haemo}$ osteoclast cultures.

Analysis of *Cre* gene expression

We performed qPCR-based analysis of the expression the Cre recombinase in osteoclasts and macrophages from the different genotypes. As expected, no Cre expression could be observed in wild type cultures. Cre expression in $Syk^{\Delta OC}$ osteoclasts was first observed two days after the initial RANKL treatment, and continued afterwards, but no increase was observed in macrophage cultures.

No Cre mRNA could be detected in $Syk^{\Delta Haemo}$ cultures which suggests that the Vav-Cre transgene is activated and Syk is deleted at an early stage of hematopoiesis.

Genetic analysis of Syk deletion during osteoclastogenesis

We performed PCR analysis on wild type, $Syk^{\Delta OC}$ and $Syk^{\Delta Haemo}$ osteoclast cultures. In wild type cultures exclusively the Syk^{+} allele was detected, and in $Syk^{\Delta Haemo}$ cells only the Syk^{Δ} variation was present, which confirms the deletion in earlier stage. On the other hand, dynamic changes are observed in $Syk^{\Delta OC}$ cultures: on the 1st day after RANKL treatment only Syk^{flox} allele is present, but in the following days Syk^{Δ} variation gradually increases while Syk^{flox} allele decreases. Taken together, those results and the time course of the changes indicate that Ctsk-Cre-mediated deletion of the Syk^{flox} allele s gradually during 2-4

days after RANKL addition and that only an incomplete genetic deletion of Syk is achieved even until the end of the observation period.

The above results indicate slow and gradual deletion of the *Syk*^{flox} allele in *Syk*$^{\Delta OC}$ osteoclast cultures, which is in line with the slow activation of the *Ctsk* gene during *in vitro* osteoclast development. These results may also explain the less severe *in vivo* phenotypes and less pronounced *in vitro* osteoclast developmental defect, as well as the continuous presence of Syk in osteoclast cultures, in the *Syk*$^{\Delta OC}$ mutants, as compared with the *Syk*$^{\Delta Haemo}$ mutants which show early and complete deletion of the *Syk*^{flox} allele from the beginning of the entire osteoclast developmental process.

The role of PI3Kβ in osteoclast function

Actin ring maintenance

Based on our previous results we supposed that PI3Kβ is required not only for the development of osteoclasts and for actin ring formation but also for the normal function of mature osteoclasts and for the maintenance of actin ring structures. To test the actin ring maintenance capability of these cells we treated wild type osteoclast by RANKL for 3 days while the mature osteoclasts developed the actin rings. We administered 50 nM PI3Kβ-specific inhibitor TGX221 or control vehicle (DMSO). During the control experiment the actin ring structures were maintained normally, while the TGX221 caused a quick fragmentation of the actin rings. These results indicate that PI3Kβ is required for the maintenance of actin ring structures.

Conclusions

Based on the results of our studies, I summarize my conclusions in the following points:

1) The osteoclast-specific deletion of Syk causes mild increase in bone density while hematopoietic deletion of Syk causes robust increase in bone density.
2) The osteoclast-specific deletion of Syk causes remarkable damage in osteoclast development and function while hematopoietic deletion of Syk entirely blocks osteoclastogenesis.
3) The osteoclast-specific deletion causes a later and incomplete deletion of Syk during osteoclast development, while the hematopoietic deletion causes an early, complete deletion of Syk, leading to major differences between the genotypes.
4) PI3Kβ is required for the maintenance of actin rings in osteoclast.

Publications

PUBLICATIONS RELEVANT TO THE DISSERTATION:

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2) 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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3.) Nemeth B, Doczi J, **Csete D**, Kacso G, Ravasz D, Adams D, Kiss G, Nagy A M, Horvath G, Tretter L, Mocsai A, Csepanyi-Komi R, Iordanov I, Adam-Vizi V, Chinopoulos C (2016) Abolition of mitochondrial substrate-level phosphorylation by itaconic acid produced by LPS-induced Irg1 expression in cells of murine macrophage lineage. *FASEB Journal*, 30 (1):286-300.
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