RANKL-independent osteoclastogenesis in the SH3BP2 cherubism mice

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Abstract

Even though the receptor activator of the nuclear factor-xB ligand (RANKL) and its receptor RANK have an exclusive role in osteoclastogenesis, the possibility of RANKL/RANK-independent osteoclastogenesis has been the subject of a long-standing debate in bone biology. In contrast, it has been reported that calvarial injection of TNF-α elicits significant osteoclastogenesis in the absence of RANKL/RANK in NF-κB2- and RBP-J-deficient mice, suggesting that inflammatory challenges and secondary gene manipulation are the prerequisites for RANKL/RANK-deficient mice to develop osteoclasts in vivo. Here we report that, even in the absence of RANKL (Rankf−/−), cherubism mice (Sh3bp2+/−/−) harboring the homozygous gain-of-function mutation in SH3-domain binding protein 2 (SH3BP2) develop tartrate-resistant acid phosphatase (TRAP)-positive multinucleated osteoclasts spontaneously. The Sh3bp2+/−/− Rankf−/− mice exhibit an increase in tooth exposure and a decrease in bone volume/total volume compared to Sh3bp2+/+/+ Rankf−/− mice. The multinucleated cells were stained positively for cathepsin K. Osteoclastic marker gene expression in bone and serum TRAP5b levels were elevated in Sh3bp2+/−/− Rankf−/− mice. Elevation of the serum TNF-α levels suggested that TNF-α is a driver for the RANKL-independent osteoclast formation in Sh3bp2+/−/− mice. Our results provide a novel mutant model that develops osteoclasts independent of RANKL and establish that the gain-of-function of SH3BP2 promotes osteoclastogenesis not only in the presence of RANKL but also in the absence of RANKL.

1. Introduction

Osteoclasts are bone-degrading multinucleated cells critical for homeostatic bone remodeling and pathologic osteolysis (Novack, 2016). Histologically, the multinucleated cells are positive for tartrate-resistant acid phosphatase (TRAP) on the bone surfaces. Even though previous studies have established that the receptor activator of nuclear factor-xB ligand (RANKL) and its receptor RANK play a crucial role in osteoclast formation, RANKL/RANK-independent osteoclastogenesis in in vivo conditions of “wild-type mice” has been a long-standing debate (Tanaka, 2017). Recently, a study suggested the ability of wild-type osteoclast precursors to differentiate into functional osteoclasts in vivo in the absence of RANK (O’Brien et al., 2016). However, it is very difficult to exclude the possibility of incomplete deletion of the Rank, because inducible Mx1-Cre system was used to delete Rank in the report (Okamoto et al., 2017). Instead, in vivo RANK/RANKL-independent osteoclastogenesis in a calvarial TNF-α injection model has been demonstrated in mice with deletion of signaling mediators such as NF-xB2 and RBP-J (Yao et al., 2009; Zhao et al., 2012).

Cherubism (OMIM#118400) is an autosomal-dominant craniofacial disorder in children characterized by expansive destruction of the maxilla and mandible. Gain-of-function mutations in the signaling adaptor SH3-domain binding protein 2 are responsible for the rare disorder (Ueki et al., 2001). We have shown that homozygous P416R knock-in (KI) mice (Sh3bp2+/−/−) harboring the most common mutation in cherubism patients recapitulate the features of human cherubism by developing spontaneous fibrous inflammatory lesions and by exhibiting increased osteoclast formation in the jaw (Ueki et al., 2007). Heterozygous and homozygous cherubism mutations promote osteoclastogenesis induced by receptor activator of nuclear factor-xB ligand (RANKL) and tumor necrosis factor-alpha (TNF-α) (Ueki et al., 2007; Mukai et al., 2014).

Here, we show that homozygous Sh3bp2+/−/− mutant mice spontaneously develop TRAP-positive (+) multinucleated osteoclasts even when RANK is absent. The Sh3bp2+/−/− Rankf−/− osteoclasts are also positive for cathepsin K. Reduced bone mass and increased levels of...
created on the mix background of C57BL/6 and 129 × 1/SvJ under Jackson laboratory (Bar Harbor, ME, USA). All mice were crossed and

2.2. Ex-vivo microCT (\(\mu\)CT) analysis

Jaw bone and femur from 20-week old mice were fixed with 4% paraformaldehyde (PFA) in PBS for 24 h and soaked in 70% ethanol for

2.3. Histology

After \(\mu\)CT analysis, mandible and femur were decalcified with EDTA (0.5 M, pH 7.2) and embedded in paraffin. Six \(\mu\)m sections were sub-

2.4. Histomorphometry of TRAP-positive osteoclasts

The number of TRAP + cells underneath the growth plate of the distal end of the femur was measured with the Skyscan 1174 (Bruker, Kontich, Belgium). Scanning conditions are as follows: 80 kV X-ray energy, 6.67 \(\mu\)m pixel size, and 0.4° rotation step with 3000 ms of exposure time. Scanned data were re-

2.5. Immunohistochemical staining

Tissue sections were deparaffinized and rehydrated, then endo-

2.6. RNA extraction from femurs

After removing soft tissues, head and distal end of the femur were cut off. The remaining part of the femur was flash frozen in liquid ni-

2.7. Reverse transcription-quantitative PCR (RT-qPCR)

Five hundred nanograms of total RNA was transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA). qPCR reactions were performed with the StepOne Plus System (Life Technologies) using Maxima SYBR green mix (Thermo Fisher Scientific, Waltham, MA, USA). qPCR primers published in our previous study were used in this study (Kittaka et al., 2020). Relative gene expression levels were calculated using a relative-

2.8. Serum ELISA

Mouse TNF-\(\alpha\) Duo-Set ELISA kit (R&D Systems, Minneapolis, MN, USA), mouse TRAP5b assay kit (Immunodiagnostic Systems, Boldon, UK), and RatLaps CTX-I EIA kit (Immunodiagnostic Systems) were used to measure serum levels of TNF-\(\alpha\), TRAP5b, and CTX. Serum was se-

3. Results

3.1. Homozygous gain-of-function mutation in SH3BP2 improves osteopetrosis in RANKL-deficient mice

We have previously reported that the gain-of-function mutation in SH3BP2 (Sh3bp2\(^{Kl/Kl}\)), which is responsible for cherubism, increases the susceptibility of bone marrow-dependent M-CSF-dependent macrophages (BMMs) to TNF-\(\alpha\) and stimulates the osteoclast formation in vivo (Mukai et al., 2014). Therefore, we hypothesized that the Sh3bp2\(^{Kl/Kl}\) mutation may improve osteopetrosis in RANKL-deficient (Rankl\(^{-/-}\)) mice by inducing osteoclasts.

To test this hypothesis, we created Sh3bp2\(^{Kl/Kl}\) Rankl\(^{-/-}\) double mutant mice. MicroCT analysis found that while the Sh3bp2\(^{Kl/Kl}\) mutation fails to exhibit tooth eruption (Fig. 1A), alveolar bone covering the tooth is significantly less than Rankl\(^{-/-}\) mice (Fig. 1B). The Sh3bp2\(^{Kl/Kl}\) mutation increased the femur length in males, but improved osteopetrosis by decreasing bone volume/total volume (BV/TV) at distal femur in male and female Rankl\(^{-/-}\) mice (Fig. 1C, D). The smaller bodyweight of Rankl\(^{-/-}\) mice was not restored by the Sh3bp2\(^{Kl/Kl}\) mutation (Fig. 1E). H&E staining confirmed that the route for tooth eruption is open more widely and that bone marrow spaces occupied by hematopoietic cells are more expanded in Sh3bp2\(^{Kl/Kl}\) Rankl\(^{-/-}\) mice than Rankl\(^{-/-}\) mice (Fig. 1F, G). These data present that the Sh3bp2\(^{Kl/Kl}\) mutation improves the osteopetrotic phenotype of

room temperature followed by treatment with Vectastain elite ABC kit (Vector Laboratories). Sections were colored using ImmPACT DAB (Vector Laboratories) and counterstained with hematoxylin.

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A. *Sh3bp2*^{+/+}*Rankl^{+/+}*, *Sh3bp2*^{+/+}*Rankl^{-/-}*, *Sh3bp2*^{+/+}*Rankl^{-/-}*

B. Male

|                | Male | Female |
|----------------|------|--------|
| *Sh3bp2*^{+/-} | *+/+* | K/I/K  |
| *Rankl*^{+/-}  | *+/+* | K/I/K  |

Exposed tooth width (%)

|                | Male | Female |
|----------------|------|--------|
| *Sh3bp2*^{+/-} | *+/+* | K/I/K  |
| *Rankl*^{+/-}  | *+/+* | K/I/K  |

C. Femur length (mm)

|                | Male | Female |
|----------------|------|--------|
| *Sh3bp2*^{+/-} | *+/+* | K/I/K  |
| *Rankl*^{+/-}  | *+/+* | K/I/K  |

D. Male

|                | Male | Female |
|----------------|------|--------|
| *Sh3bp2*^{+/-} | *+/+* | K/I/K  |
| *Rankl*^{+/-}  | *+/+* | K/I/K  |

BV/TV (%)

|                | Male | Female |
|----------------|------|--------|
| *Sh3bp2*^{+/-} | *+/+* | K/I/K  |
| *Rankl*^{+/-}  | *+/+* | K/I/K  |

E. Body weight (g)

|                | Male | Female |
|----------------|------|--------|
| *Sh3bp2*^{+/-} | *+/+* | K/I/K  |
| *Rankl*^{+/-}  | *+/+* | K/I/K  |

F. *Sh3bp2*^{+/+}*Rankl^{+/+}*, *Sh3bp2*^{+/+}*Rankl^{-/-}*, *Sh3bp2*^{+/+}*Rankl^{-/-}*

G. *Sh3bp2*^{+/+}*Rankl^{+/+}*, *Sh3bp2*^{+/+}*Rankl^{-/-}*, *Sh3bp2*^{+/+}*Rankl^{-/-}*

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Fig. 1. (A) Representative images showing incisor eruption. Arrowheads indicate erupted incisors in the wild-type mouse at 20 weeks old. (B) Left top: 3D μCT images of the mandibular molars. Left bottom: 2D coronal μCT images at the yellow line. Right: Percentage of opening width of the tooth measured at the midpoints of the lower third molar. (C) Left: 3D μCT images of the femur. Right: length of the femur. (D) Left: 2D sagittal μCT images of the femur. Right: Bone volume (BV)/ tissue volume (TV) of trabecular bone. (E) Body weight of the mice. (F) H&E staining of the mandibular third molar. Bar = 100 μm. (G) H&E staining of the femur. Boxes represent the area shown at higher magnification. Mice were analyzed at 20 weeks old. All images are representative from male mice. Data are mean ± SD. Each dot in graphs represent a value from a single biological sample. p values in graphs were calculated by one-way ANOVA with Tukey's multiple comparison test. p < 0.05 was considered to be significantly different.

3.2. RANKL-deficient Sh3bp2KO/ KO mice develop TRAP+ osteoclasts

To examine whether Sh3bp2KO/ KO Rankl+/− mice develop osteoclasts in vivo, we performed TRAP staining on alveolar and trabecular bone sections from the mandible and femur of the Sh3bp2KO/ KO Rankl+/− mice. Although the overall TRAP-positive (+) osteoclast number was much smaller than Sh3bp2+/+Rankl+/− mice, a number of TRAP+ multinucleated osteoclasts were developed on the bone surface of Sh3bp2KO/ KO Rankl+/− mice (Fig. 2A, B). The number was increased toward the growth plate in male Sh3bp2KO/ KO Rankl+/− mice (Fig. 2C). The TRAP+ multinucleated cells were stained positively for an osteoclast marker cathepsin K (Fig. 2D). Consistent with the presence of TRAP+ and cathepsin K+ cells, expression levels of osteoclast maker genes such as Acp5, Cathepsin K, and Mmp9 were increased in the femur of Sh3bp2KO/ KO Rankl−/− mice compared to Sh3bp2+/+Rankl+/− mice (Fig. 2E). Serum TRAP5b levels were elevated in Sh3bp2KO/ KO Rankl−/− mice and serum CTX levels were increased in female Sh3bp2KO/ KO Rankl−/− mice compared to Sh3bp2+/+Rankl+/− mice (Fig. 2F). Consistent with our previous results, Sh3bp2KO/ KO mutation increased serum TNF-α levels in Rankl−/− mice (Fig. 2G). In contrast, RANKL deletion suppressed the elevation of serum TNF-α in Sh3bp2KO/ KO cherubism mice (Fig. 2G). Together, the data suggest that Sh3bp2KO/ KO Rankl+/−/− osteoclasts are functionally active and that TNF-α substitutes for RANKL to develop TRAP+ osteoclasts in Sh3bp2KO/ KO Rankl+/− mice.

4. Discussion

We have previously shown that bone marrow-derived M-CSF-dependent macrophages harboring a gain-of-function mutation in SH3BP2 are highly susceptible to TNF-α, and they can form TRAP+ osteoclasts independent of RANKL in vitro (Mukai et al., 2014). In this study, we showed that the Sh3bp2KO/ KO mutation can develop TRAP+ multinucleated osteoclasts in RANKL-deficient mice spontaneously. It also improved the osteopetrotic phenotype of the mice, presumably cooperated with impaired osteoclast function of Sh3bp2KO/ KO mice (Mukherjee et al., 2010; Wang et al., 2010).

To our best knowledge, RANKL-deficient Sh3bp2KO/ KO mice are the third model that develops RANK/RANKL-independent osteoclasts after the Nfkb2−/− and RbpjΔ/MΔ mice injected with TNF-α on the calvaria (Yao et al., 2009; Zhao et al., 2012). However, they are the first human disease model that exhibits RANK/RANKL-independent osteoclastogenesis. Also, Sh3bp2KO/ KO Rankl+/− mice are the second model that shows the spontaneous osteoclast development in the absence of the RANK/RANKL signaling following the RbpjΔ/MΔ mice, although Zhao et al. did not clarify whether TRAP+ cells in the calvaria of PBS-injected Rankl−/−/ RbpjΔ/MΔ mice are osteoclasts or not (in the Fig. 3F of (Zhao et al., 2012)).

Even though the possibility of RANKL/RANK-independent osteoclastogenesis in wild-type mice has been a long-standing debate, many cell culture studies have suggested the RANK/RANK-independent osteoclastogenesis of wild-type osteoclast precursors (Tanaka, 2017; Okamoto et al., 2017). Notably, Kim et al. showed that M-CSF/TGF-β-dependent osteoclast precursors from RANKL- and RANK-deficient mice are able to form functionally active osteoclasts when stimulated with TNF-α and IL-1 (IL-1 types are not specified in the report) (Kim et al., 2005). However, the consequences of TNF-α and IL-1 stimulation in vivo were not explored in this study.

A recent study by O’Brien et al. showed TRAP+ osteoclast formation in the K/BxN serum-transfer arthritis model, in which RANK is deleted by Mx1-Cre (O’Brien et al., 2016). However, it is difficult to exclude the possibility of incomplete deletion of the RANK gene, because Cre is inducibly expressed in type I interferon receptor (IFNAR)-expressing cells after polyinosine-polycytidylic acid (pIpc) injection (Okamoto et al., 2017; Kuhn et al., 1995). Therefore, the current consensus on RANK/RANKL-independent osteoclastogenesis is that additional gene manipulation is required for RANK/RANKL-deficient mice to develop osteoclasts in vivo. Because Sh3bp2KO/ KO Rankl−/− mice show not only a decrease in BV/TV but also an increase in bone resorption markers in serum compared to Sh3bp2+/+ Rankl+/− mice, homozygous Sh3bp2KO/ KO mutation serves as a new genetic manipulation to enable the formation of functional osteoclasts in the absence of RANKL.

We have previously shown that retrolental overexpression of wild-type and cherubism mutant form of SH3BP2 in RAW264.7 cells induces TRAP+ multinucleated cells without RANKL stimulation (Leavao et al., 2011). We have also shown that TNF-α stimulation in the presence of SH3BP2 gain-of-function can induce osteoclastogenesis via the SYK-NFATc1 axis (Mukai et al., 2014). These results suggest that activation of the downstream signaling of SH3BP2 play a crucial role in RANKL-independent osteoclast formation. Since the gain-of-function of SH3BP2 increases SYK activation in myeloid cells (Ueki et al., 2007; Mukai et al., 2014; Yoshitaka et al., 2014), SYK is likely involved in the mechanism of RANKL-independent osteoclastogenesis. Pathological conditions caused by the hyperactivation of SYK in myeloid lineage cells (Puissant et al., 2014; Mocsai et al., 2010; Ozaki et al., 2012) may exhibit bone loss independent of RANKL, at least in part. Individuals with loss-of-function mutations in RANK or RANKL exhibit osteopetrosis (Guerrini et al., 2008; Sobacchi et al., 2007). Because tankyrase inhibitors can activate the SH3BP2-SYK pathway in osteoclasts by preventing SH3BP2 protein from degradation (Fujita et al., 2018), patients with certain types of osteopetrosis may benefit from tankyrase inhibitor treatment by inducing RANK/RANKL-independent osteoclastic bone resorption.

Elevation of the serum CTX in Sh3bp2KO/ KO Rankl−/− mice was significant in females, but not in males. The gender-dependent effect may be explained by the possibility that not all Sh3bp2KO/ KO Rankl−/− TRAP+ cells have active bone resorption capacity in males. In addition, a supplemental analysis revealed that male Sh3bp2KO/ KO Rankl−/− mice show the increase in serum CTX (p < 0.01) if an outlier is excluded in each group (Grubbs' method with α = 0.05).

Intriguingly, lack of RANKL decreased the serum TNF-α elevation in Sh3bp2KO/ KO cherubism mice. This result suggests that the RANK-mediated signaling in macrophages plays a critical role in the development or exacerbation of autoinflammation in cherubism mice. Together, RANKL inhibitors might be beneficial for suppressing the development of lesions in cherubism patients. These hypotheses need to be validated in further studies.

In conclusion, we showed that the cherubism mice carrying the gain-of-function mutation in SH3BP2 develop osteoclasts in a RANKL-independent manner. These results will reinforce our current understanding that SH3BP2 is an adaptor protein critical for the regulation of osteoclastogenesis and that additional gene manipulation is necessary.
Fig. 2. (A) TRAP staining of alveolar bone surrounding the mandibular third molar. The boxes represent the area shown at higher magnification. Arrowheads indicate TRAP+ cells on the bone surface. (B) TRAP staining of trabecular bone of the femur. Boxes represent the area shown at higher magnification. Arrowheads indicate TRAP+ cells on the bone surface. (C) Left: An image showing the ROI. Middle: The number of TRAP+ osteoclasts/mm² in each ROI (a, b, c). Right: The number of TRAP+ osteoclasts/mm² from Rankl−/− mice. (D) Immunohistochemistry of cathepsin K. Boxes represent the area shown at higher magnification. (E) qPCR analysis of osteoclast maker gene expression in the femur. Average expression levels in the wild-type mice were set as 1. (F) Serum levels of TRAP5b and CTX. (G) Serum levels of TNF-α. Mice were analyzed at 20 weeks old. All images are representative from male mice. Data are mean ± SD. Each dot in graphs represents a value from a single biological sample. p values in graphs were calculated by one-way ANOVA with Tukey’s multiple comparison test (C) or t-test (E, F, G). p < 0.05 was considered to be significantly different.
for achieving osteoclastogenesis in the absence of RANK/RANKL.

**Authors’ contribution**

MK and YU conceived the idea and initiated the project. MK generated the mice. MK, TY, HH, and MEL conducted analyses and contributed to the data interpretation. YU wrote the manuscript, and all authors approved the final version of the manuscript. MK and YU take responsibility for the integrity of the data and analysis.

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**Declaration of competing interest**

The authors have no conflict of interest to declare.

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