Biochemical and Biophysical Research Communications 498 (2018) 967–974

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Dok-3 and Dok-1/-2 adaptors play distinctive roles in cell fusion and proliferation during osteoclastogenesis and cooperatively protect mice from osteopenia

Shuhei Kajikawa [a], Yuu Taguchi [b], Tadayoshi Hayata [c, 1], Yoichi Ezura [c, 2], Ryo Ueta [a], Sumimasa Arimura [a], Jun-ichiro Inoue [b], Masaki Noda [c, 3], Yuji Yamanashi [a, *]

[a] Division of Genetics, The Institute of Medical Science, The University of Tokyo, Shirokanedai, Minato-ku, Tokyo, 108-8639, Japan
[b] Division of Cellular and Molecular Biology, The Institute of Medical Science, The University of Tokyo, Shirokanedai, Minato-ku, Tokyo, 108-8639, Japan
[c] Department of Molecular Pharmacology, Medical Research Institute, Tokyo Medical and Dental University, Yushima, Bunkyo-ku, Tokyo, 113-8510, Japan

ARTICLE INFO

Article history:
Received 21 February 2018
Accepted 13 March 2018
Available online 17 March 2018

Keywords:
Dok
Osteoclastogenesis
Cell-to-cell fusion
Osteopenia

ABSTRACT

Bone mass is determined by coordinated acts of osteoblasts and osteoclasts, which control bone formation and resorption, respectively. Osteoclasts are multinucleated, macrophage/monocyte lineage cells from bone marrow. The Dok-family adaptors Dok-1, Dok-2 and Dok-3 are expressed in the macrophage/monocyte lineage and negatively regulate many signaling pathways, implying roles in osteoclastogenesis. Indeed, mice lacking Dok-1 and Dok-2, the closest homologues with redundant functions, develop osteopenia with increased osteoclast counts compared to the wild-type controls. Here, we demonstrate that Dok-3 knockout (KO) mice also develop osteopenia. However, Dok-3 KO, but not Dok-1/-2 double-KO (DKO), mice develop larger osteoclasts within the normal cell-count range, suggesting a distinctive role for Dok-3. Indeed, Dok-3 KO, but not Dok-1/-2 DKO, bone marrow-derived cells (BMDCs) generated larger osteoclasts with more nuclei due to augmented cell-to-cell fusion in vitro. In addition, while Dok-1/-2 DKO BMDCs generated more osteoclasts, Dok-1/-2/-3 triple-KO (TKO) BMDCs generated osteoclasts increased in both number and size. Furthermore, Dok-1/-2/-3 TKO mice showed the combined effects of Dok-3 and Dok-1/-2 deficiency: severe osteopenia with more and larger osteoclasts. Together, our findings demonstrate that Dok-3 and Dok-1/-2 play distinctive but cooperative roles in osteoclastogenesis and protect mice from osteopenia, providing physiological and pathophysiological insight into bone homeostasis.

1. Introduction

The bone is essential for structural support of the body and protection of internal organs, and also provides mineral storage for metabolic homeostasis. Bone mass is regulated in a fine-tuned balance between bone formation and resorption [1], and breakdown of this balance leads to bone disorders characterized by altered bone mass, such as osteoporosis, Paget's disease of bone and osteopetrosis [2–4]. It has been established that osteoblasts and osteoclasts play critical roles in bone formation and resorption, respectively, and indeed dysfunction of these cells is involved in pathogenesis of altered bone mass [2–4].

Osteoclasts develop from the macrophage/monocyte lineage in bone marrow while osteoblasts derive from mesenchymal progenitors. Osteoclastogenesis consists of multiple steps, including 1) recruitment of osteoclast progenitors from the hematopoietic lineage, 2) proliferation of the progenitors, 3) differentiation of the progenitors to precursors, 4) proliferation of the precursors, 5) differentiation of the precursors to tartrate-resistant acid phosphatase (TRAP)-positive mononucleated osteoclasts, and 6) cell-to-cell fusion of the mononucleated osteoclasts to become fully differentiated, TRAP-positive ($TRAP^+$) multinucleated osteoclasts

* Corresponding author.
E-mail address: yyamanas@ims.u-tokyo.ac.jp (Y. Yamanashi).
[1] Department of Biological Signaling and Regulation, Faculty of Medicine, University of Tsukuba, Tennodai, Tsukuba, Ibaraki, 305-8550, Japan.
[2] Department of Frontier Research Unit Skeletal Molecular Pharmacology, Medical Research Institute, Tokyo Medical and Dental University, Yushima, Bunkyo-ku, Tokyo, 113-8510, Japan.
[3] Yokohama City Minato Red Cross Hospital, Shinyamashita, Naka-ku, Yokohama, Kanagawa, 231-8682, Japan.

https://doi.org/10.1016/j.bbrc.2018.03.090
0006-291X/

[5–7]. This process is often investigated in vitro by utilizing two pivotal osteoclastogenic cytokines, the macrophage colony-stimulating factor (M-CSF) and receptor activator for nuclear factor-κB ligand (RANKL) [5–7]. In general, M-CSF promotes proliferation and survival of osteoclast precursors, whereas RANKL induces osteoclast differentiation, including cell-to-cell fusion that is critical for the formation of TRAP$^+$ multinucleated osteoclasts [8,9]. However, the regulation of M-CSF- and RANKL-mediated signaling in osteoclastogenesis remains unclear.

The Downstream of tyrosine kinases (Dok)-family includes seven proteins, Dok-1 to Dok-7, which share structural similarities characterized by the NH2-terminal pleckstrin homology and phosphotyrosine-binding domains followed by SH2 target motifs in the COOH-terminal portion [10]. Among these members, only Dok-1, Dok-2 and Dok-3 are expressed preferentially in hematopoietic cells, including macrophages and monocytes, and comprise a closely related subgroup with regard to their primary structure. Accumulating evidence indicates that Dok-1, Dok-2 and Dok-3 suppress proliferation, survival and cytokine production in hematopoietic cells [10–15]. Previously, we demonstrated that mice lacking Dok-1 and its closest homolog Dok-2 [Dok-1/-2 double-knockout (DKO) mice] developed osteopenia with enhanced bone resorption phenotypes, including an increased number of osteoclasts, which are generated from the macrophage/monocyte lineage of hematopoietic cells. Thus, we concluded that Dok-1 and Dok-2 negatively regulate bone resorption and osteoclastogenesis [16]. However, any involvement of Dok-3 in these events remains to be studied.

Here, we analyzed the bone phenotypes in Dok-3 KO and Dok-1, Dok-2 and Dok-3 triple-KO (Dok-1/-2/-3 TKO) mice, in comparison with those in Dok-1/-2 DKO and wild-type (WT) mice on a C57BL/6 genetic background. In addition, possible roles for Dok-3 and Dok-1/-2 were further investigated in osteoclastogenesis in vitro. Our findings demonstrate that Dok-3 and Dok-1/-2 play distinctive roles in osteoclastogenesis and cooperatively protect mice from osteopenia. More precisely, Dok-3 inhibits osteoclastogenesis preferentially by suppressing RANKL-mediated cell-to-cell fusion of osteoclasts while Dok-1/-2 inhibits osteoclastogenesis preferentially by suppressing M-CSF-mediated proliferation of osteoclast precursors.

2. Materials and methods

This section is described in Supplementary Information (Supplementary Materials and Methods).

3. Results

3.1. Mice lacking Dok-3 and/or Dok-1/-2 show decreased bone mass

To investigate bone phenotypes in Dok-3 KO and Dok-1/-2/-3 TKO mice in comparison to those in Dok-1/-2 DKO and WT mice, micro-computed tomography (μCT) and bone histomorphometric analyses were performed at 9 weeks of age with the distal femur of male mice, given that male trabecular bone mass of the distal femur reaches its peak value at 2 months of age [17]. The μCT analysis revealed that compared to WT mice, the trabecular bone mass was decreased in Dok-3 KO and Dok-1/-2/-3 TKO mice, and the decrement levels were similar and enhanced, respectively, to that observed in Dok-1/-2 DKO mice (Fig. 1A and B). Decreased trabecular bone thickness (Tb.Th) and number (Tb.N) were associated with increased trabecular bone separation (Tb.Sp) and spacing (Tb.Spac) in all the mutant mice (Fig. 1C–F). Histological analysis of the distal femoral metaphysis with hematoxylin and eosin (H&E) staining indicated a consistent decrease in the trabecular bone mass in these mutant mice (Fig. 1G). Together, these data demonstrate that Dok-3 KO mice develop osteopenia like Dok-1/-2 DKO mice. In addition, the exacerbated pathology found in Dok-1/-2/-3 TKO mice further demonstrate that Dok-3 and Dok-1/-2 cooperatively protect mice from osteopenia.

3.2. Dok-3 KO and Dok-1/-2 DKO mice develop larger and more numerous osteoclasts, respectively

In general, decreased bone mass is associated with decreased osteoblastic bone formation and/or increased osteoclastic bone resorption. Thus, we investigated the following dynamic bone formation parameters in trabecular bone at the distal femur: 1) mineralizing surface per bone surface (MS/BS), 2) mineral apposition rate (MAR), and 3) bone formation rate (BFR). We found no significant difference between Dok-mutant and WT mice at 6 weeks of age, when bone turnover by osteoblasts and osteoclasts is prominent in mice on a C57BL/6J background (Supplementary Fig. S1A–C) [18,19]. Consistent with this, the number of osteoblasts per bone surface and the osteoblast surface per bone surface were also comparable to each other (Supplementary Fig. S1D and E). In addition, mineralized nodule formation by osteoblasts in bone marrow cells from Dok-mutant or WT mice showed no significant difference (Supplementary Fig. S1F and G). Together, these findings demonstrate no obvious defects in bone formation in Dok-mutant mice at 6 weeks of age. Thus, we further investigated bone resorption parameters based on TRAP staining of the histological sections and found a significantly increased number of osteoclasts on the trabecular surface of Dok-1/-2 DKO, but not Dok-3 KO, mice as compared to that of WT mice at 6 weeks of age (Fig. 2A and C). By contrast, we found abnormally large osteoclasts in Dok-3 KO, but not Dok-1/-2 DKO, mice (Fig. 2A, B and D). These results suggest that Dok-3 and Dok-1/-2 play distinctive roles in osteoclastogenesis. However, given that Dok-1/-2/-3 TKO mice develop much larger osteoclasts than Dok-3 KO mice (Fig. 2A, B and D), our findings also imply that Dok-1/-2 may play a partially overlapping role with Dok-3 in osteoclastogenesis.

3.3. Bone marrow-derived cells from mice lacking Dok-3 generate enlarged osteoclasts in vitro

To address pathological bases for the osteoclast abnormality in Dok-mutant mice, we performed in vitro osteoclastogenesis assays. Bone marrow-derived cells (BMDCs) were prepared from femora and tibiae as described in Materials and Methods and cultured in the presence of M-CSF and RANKL, which generally promote proliferation/survival and differentiation of osteoclast precursors in BMDCs, respectively. We first confirmed that the number of TRAP$^+$ multinucleated ($\geq$3 nuclei) osteoclasts per well was increased with Dok-1/-2 DKO BMDCs as compared to the WT controls, as we had previously reported (Fig. 3A and B) [16]. In addition, we found that the total number of nuclei per well, in which an equal number of BMDCs were plated, was also increased in Dok-1/-2 DKO BMDCs as compared to the WT controls (Fig. 3A and C), suggesting an enhanced proliferation of osteoclast precursors because the precursor population in bone marrow cells were comparable between Dok-mutant and WT mice as further described below (Supplementary Fig. S2). However, although Dok-3 KO BMDCs generated a slightly increased number of TRAP$^+$ multinucleated osteoclasts per well, the total number of nuclei per well was not significantly increased as compared to the WT controls (Fig. 3A–C), suggesting an enhancement of osteoclast differentiation, including cell-to-cell fusion. Indeed, the size and nucleus number of TRAP$^+$ multinucleated Dok-3 KO osteoclasts were increased compared to WT or Dok-1/-2 DKO cells (Fig. 3A, D and E). Although Dok-1/-2