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Necrostatin-7 suppresses RANK-NFATc1 signaling and attenuates macrophage to osteoclast differentiation

Hiroaki Fuji [a, 1], Saori Ohmae [b, 1], Naruto Noma [c, 1], Masatoshi Takeiri [c, 1], Hideto Yasutomi [d], Kazuya Izumi [d], Moe Ito [d], Masayasu Toyomoto [e], Soichiro Iwaki [d], Kenji Takemoto [f], Satoru Seo [a], Kojiro Taura [a], Shigeaki Hida [g], Mineyoshi Aoyama [d], Yasushi Ishihama [h], Masatoshi Hagiwara [e], Norihiko Takeda [i], Etsuro Hatano [j], Keiko Iwaisako [a, k, *], Shinji Uemoto [a], Masataka Asagiri [a, d, **]

[a] Department of Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan
[b] Department of Bioimaging and Cell Signaling, Graduate School of Biostudies, Kyoto University, Kyoto, Japan
[c] Innovation Center for Immunoregulation and Therapeutics, Graduate School of Medicine, Kyoto University, Kyoto, Japan
[d] Department of Pathobiology, Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya, Japan
[e] Department of Anatomy and Developmental Biology, Graduate School of Medicine, Kyoto University, Kyoto, Japan
[f] Center for Cell Death, Injury and Regeneration, Department of Drug Discovery & Biomedical Sciences, Medical University of South Carolina, Charleston, SC, USA
[g] Department of Molecular and Cellular Health Sciences, Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya, Japan
[h] Department of Molecular and Cellular BioAnalysis, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan
[i] Department of Cardiovascular Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan
[j] Department of Surgery, Hyogo College of Medicine, Nishinomiya, Japan
[k] Department of Medical Life Systems, Faculty of Life and Medical Sciences, Doshisha University, Kyotanabe, Japan

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ABSTRACT

Osteoclasts play a crucial role in osteolytic bone diseases, such as osteoporosis, rheumatoid arthritis, periodontitis, Paget's disease of bone and bone metastatic tumors. Therefore, controlling osteoclast differentiation and function has been considered a promising therapeutic strategy. Here, we show that necrostatin (Nec)-7, an inhibitor of programmed necrosis, strongly suppressed receptor activator of nuclear factor (NF)-κB ligand (RANKL)-induced osteoclastogenesis and bone resorption, without compromising macrophage colony-stimulating factor (M-CSF)-supported survival and growth of osteoclast precursor cells. Accordingly, Nec-7 significantly decreased the levels of RANKL-induced osteoclastogenic marker genes, such as cathepsin K. Mechanistically, Nec-7 neither affected MAPK nor NF-κB activation; however, it strongly inhibited the RANKL receptor (RANK) to nuclear factor of activated T cells c1 (NFATc1) signaling. Lentiviral expression of RANK in bone marrow-derived macrophages significantly restored osteoclastogenesis and NFATc1 amplification in Nec-7-treated cells. In this study, we revealed that Nec-7-sensitive pathways are crucially involved in osteoclast formation and function. Investigation of the molecular mechanism(s) through which Nec-7 inhibits RANK-NFATc1 signaling axis may lead to the development of new therapeutic strategies for bone disease.

Abbreviations: RANK, receptor activator of nuclear factor-kappa B; RANKL, RANK ligand; M-CSF, macrophage colony-stimulating factor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor-kappa B; IκBα, s; NFATc1, nuclear factor of activated T cells c1; Acp5, acid phosphatase, 5 tartrate resistant; TRAP, tartrate-resistant acid phosphatase; Atp6v0d2, ATPase H+ transporting, lysosomal V0 subunit D2; Ctsk, cathepsin K; Dcstamp, dendrocyte expressed seven transmembrane protein; Nec, necrostatin; BMM, bone marrow-derived macrophage.

* Corresponding author. 1-3 Tatara Miyakodani, Kyotanabe, Kyoto, 610-0394, Japan.
** Corresponding author. 3-1 Tanabe-dori, Mizuho-ku, Nagoya, 467-8603, Japan.
E-mail addresses: iwaisako@kuhp.kyoto-u.ac.jp (K. Iwaisako), masa-asagiri@umin.org (M. Asagiri).
[1] These authors contributed equally to this work.

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1. Introduction

Throughout life, the bone is constantly renewed, and its strength and integrity are maintained by the coordination of osteoclast-mediated bone resorption and osteoblast-mediated bone formation [1,2]. Osteoclasts are multi-nucleated giant cells formed by the fusion and differentiation of the hematopoietic monocyte/macrophage lineage, and when activated, they degrade bone matrix by secreting protons and lysosomal proteases [2,3]. Excessive bone resorption has been shown to be involved in a variety of bone disorders such as osteoporosis, rheumatoid arthritis, Paget's disease of bone, and cancer metastasis to bone [2,4,5], and patients affected by these conditions suffer from fracture, pain and impaired mobility [6–8]. Therefore, osteoclastic bone resorption has been considered a therapeutic target, and in fact, some drugs, such as bisphosphonates and denosumab (a monoclonal antibody against receptor activator of nuclear factor-κB ligand [RANKL]), which target osteoclasts, have been already clinically used as antiresorptive agents [9,10].

Macrophage colony-stimulating factor (M-CSF) and RANKL are two essential cytokines regulating the development and function of osteoclasts [2,11]. M-CSF mainly contributes to the survival and proliferation of osteoclast precursor cells, while RANKL enables the dynamic differentiation processes including cell-cell fusion through its receptor (RANK) [2,11–13]. Upon RANKL binding to its receptor RANK, a variety of downstream signaling pathways responsible for osteoclast differentiation are activated. Among them, especially in the early phase of osteoclastogenesis (within minutes to an hour after RANKL-RANK engagement), the activation of mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK) [1,14–16], and the activation of nuclear factor-kappa B (NF-κB) molecules [17,18], whose liberation and activation require degradation of inhibitor of κB (IκB) protein [19], are strongly induced by RANK-mediated signaling [2,20].

The early phase osteoclastogenic signaling, governed by factors such as MAPKs and NF-κB, culminates in the activation of nuclear factor of activated T cells c1 (NFATc1), a master regulator for osteoclastogenesis [2,20–22]. In the late phase of osteoclastogenesis (within hours to days after RANKL-RANK engagement), NFATc1 has been shown to bind to its own promoter and activate an autoregulatory transcriptional loop (thus, called the autoamplification of NFATc1) [2,22,23]. Then, NFATc1 transcriptionally controls osteoclast terminal differentiation by activating osteoclastogenic genes, such as *Acp5* (encoding TRAP: a marker for osteoclastogenesis), *Atp6v0d2* (encoding vacuolar-type ATPase, H^+ transporting V0 subunit D2: a component of a proton pump which contributes to the extracellular acidification of osteoclasts), *Ctsk* (encoding cathepsin K: a key cysteine protease expressed in osteoclasts), and *Dcstamp* (encoding DC-STAMP: a molecule involved in cell-cell fusion during osteoclastogenesis) over time [2,20,24,25].

Necrostatin-7 (5-[[3-(4-Fluorophenyl)-1H-pyrazol-4-yl]methylene]-2-imino-3-(2-thiazolyl)-4-thiazolidinone; CAS registry number: 351062-08-3) (Nec-7) is a chemical compound originally identified as an inhibitor of programmed necrosis (called necroptosis; which is caused by a regulated cell death mechanism that results in morphological features resembling necrosis [26,27]) in *Fas-associated protein with death domain (FADD)*-deficient Jurkat T cells [28]. However, the mechanism of action of how Nec-7 inhibits necroptosis remains totally unknown [28], whereas it has been previously reported that Nec-1, another type of necroptosis inhibitor, prevents necroptosis by inhibiting kinase activity of receptor-interacting serine/threonine-protein kinase 1 (RIPK1) [29].

In this study, we investigated the pharmacological effects of Nec-7, other than necroptosis inhibition, and found that Nec-7 potently inhibited osteoclast differentiation and function. Elucidation of the mechanism underlying its action will provide new insights into bone and osteoclast biology.

2. Materials and methods

For detailed materials and methods, see Appendix A. Supplementary Information.

3. Results

3.1. Nec-7 attenuates osteoclast differentiation and osteoclastic bone resorption in vitro

To determine the effect of Nec-7 on osteoclast differentiation, primary bone marrow-derived macrophages (BMMs) were treated with various concentrations of Nec-7 (0.1–3 μM) in the presence of M-CSF and RANKL for three days. As shown in Fig. 1A and B, Nec-7 reduced the number of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated osteoclasts in a dose-dependent manner, whereas vehicle-treated BMMs efficiently underwent osteoclastogenesis. The attenuated osteoclast differentiation could be caused by inhibitory effects of Nec-7 on the proliferation and/or survival of osteoclast precursor cells. To test this possibility, BMMs were cultured with Nec-7 for three days, and we found that Nec-7 did not show significant cytotoxicity in BMMs at concentrations up to 3 μM (Supplementary Fig. S1). In agreement with the significant attenuation of osteoclast differentiation, Nec-7 dose-dependently suppressed osteoclastic bone resorption *in vitro* (Fig. 1C and D).

Nec-7 was originally identified as an inhibitor of necroptosis [28]. To address whether the necroptosis mechanisms are generally involved in macrophage to osteoclast differentiation, we tested the effect of another type of necroptosis inhibitor, Nec-1 [29] on osteoclastogenesis. As shown in Fig. 1E, Nec-1 (up to 10 μM) did not influence osteoclast differentiation, whereas even 2 μM of Nec-1 significantly suppressed necroptosis in BMMs (Supplementary Fig. S2), indicating that the Nec-7-sensitive pathways responsible for osteoclastogenesis are, at least, distinct from the Nec-1-sensitive necroptosis pathway(s).

3.2. Nec-7 suppresses RANKL-stimulated osteoclastogenic gene induction

M-CSF and RANKL are essential cytokines for osteoclastogenesis, and M-CSF has been shown to support the survival and proliferation of osteoclast precursor cells [2,23]. The fact that Nec-7 did not affect survival and proliferation of BMMs (Supplementary Fig. S1) strongly suggested that Nec-7 abrogates RANKL-induced signaling rather than M-CSF-activated signaling. In fact, Nec-7 suppressed osteoclastogenesis in the murine monocyte/macrophage cell line RAW264 (Supplementary Fig. S3), which only requires RANKL but not M-CSF supplementation for osteoclastogenesis in culture [30], further supporting the notion that Nec-7 inhibits RANKL-RANK signals. We then examined the effects of Nec-7 on the RANKL-stimulated gene induction profiles in BMMs. As expected, Nec-7 dose-dependently suppressed the expression levels of *Acp5*, *Atp6v0d2*, *Ctsk* and *Dcstamp* (Fig. 2). Collectively, these data clearly demonstrate that RANK downstream signaling contains Nec-7-sensitive elements crucial for osteoclast terminal differentiation.

3.3. Nec-7 affects the RANKL-stimulated autoamplification of NFATc1 and sustained expression of RANK during osteoclastogenesis rather than the early activation of MAPKs and NF-κB

As important cellular events leading to osteoclastogenesis, the

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