Bone 92 (2016) 85–93

Contents lists available at ScienceDirect

Bone

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

Full Length Article

Parathyroid hormone induces expression and proteolytic processing of Rankl in primary murine osteoblasts

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ARTICLE INFO

Article history:
Received 25 April 2016
Revised 16 August 2016
Accepted 18 August 2016
Available online 20 August 2016

Keywords:
Adamts
Ectodomain shedding
Osteoblast
PTH
Rankl

ABSTRACT

Rankl, the major pro-osteoclastogenic cytokine, is synthesized as a transmembrane protein that can be cleaved by specific endopeptidases to release a soluble form (sRankl). We have previously reported that interleukin-33 (IL-33) induces expression of *Tnfsf11*, the Rankl-encoding gene, in primary osteoblasts, but we failed to detect sRankl in the medium. Since we also found that PTH treatment caused sRankl release in a similar experimental setting, we directly compared the influence of the two molecules. Here we show that treatment of primary murine osteoblasts with PTH causes sRankl release into the medium, whereas IL-33 only induces *Tnfsf11* expression. This difference was not explainable by alternative splicing or by PTH-specific induction of endopeptidases previously shown to facilitate Rankl processing. Since sRankl release after PTH administration was blocked in the presence a broad-spectrum matrix metalloprotease inhibitor, we applied genome-wide expression analyses to identify transcriptional targets of PTH in osteoblasts. We thereby confirmed some of the effects of PTH established in other systems, but additionally identified few PTH-induced genes encoding metalloproteases. By comparing expression of these genes following administration of IL-33, PTH and various other *Tnfsf11*-inducing molecules, we observed that PTH was the only molecule simultaneously inducing sRankl release and *Adamts1* expression. The functional relevance of the putative influence of PTH on Rankl processing was further confirmed *in vivo*, as we found that daily injection of PTH into wildtype mice did not only increase bone formation, but also osteoclastogenesis and sRankl concentrations in the serum. Taken together, our findings demonstrate that transcriptional effects on *Tnfsf11* expression do not generally trigger sRankl release and that PTH has a unique activity to promote the proteolytic processing of Rankl.

1. Introduction

One of the most important regulatory systems controlling bone remodeling involves receptor activator of nuclear factor κB (Rank), its ligand (Rankl) and the Rankl decoy receptor osteoprotegerin (Opg) [1]. More specifically, Rankl expressed by cells of the osteoblast lineage is known to activate osteoclastogenesis from hematopoietic progenitors after binding to Rank in a Traf6-dependent manner [2]. The physiological relevance of the Rankl-Rank interaction is probably best underscored by the fact that mutations in either gene causes osteoclast-poor osteopetrosis in humans [3]. Likewise, the therapeutic relevance of the system is highlighted by the fact that Denosumab, a monoclonal antibody neutralizing human RANKl, has been successfully introduced as an *anti*-resorptive drug for the treatment of individuals with osteoporosis or osteolytic bone destruction [4]. Molecularly, the Rankl-Rank-Opg-system has been extensively studied through a combination of mouse genetics and tissue culture experiments. Interestingly however, one aspect of Rankl biology is still not fully understood, as Rankl is synthesized as a type II transmembrane protein that can be proteolytically processed to generate a soluble form (sRankl) [5]. In fact, since the majority of published studies either analyzed expression of the Rankl-encoding gene (*i.e. Tnsfsf11*) or the extracellular presence of soluble Rankl (sRankl), the question about its proteolytical processing was not generally addressed. Moreover, since the previously identified

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http://dx.doi.org/10.1016/j.bone.2016.08.016
8756-3282/

cleavage sites for putative Rankl sheddases (Adam10, Adam17 or Mmp14) are all located within a region of the murine Rankl protein that is not conserved in human RANKl (Fig. S1), it is at least debatable, if the findings obtained by forced expression studies are physiologically relevant [6–8].

Our interest in this particular question was triggered by a previous study, where we analyzed the molecular effects of the cytokine IL-33 on bone remodeling. More specifically we found that IL-33 acts as a potent inhibitor of osteoclastogenesis, both *in vitro* and *in vivo* [9,10]. The physiological relevance of this finding was supported by the analysis of mice lacking the IL-33 receptor, which displayed increased osteoclast indices, assessed by cellular histomorphometry on bone sections [9]. In the context of this study we additionally analyzed the influence of IL-33 on primary osteoblasts. Whereas chronic IL-33 administration during the course of differentiation did not interfere with matrix mineralization, a short-term treatment of primary osteoblasts with IL-33 induced the expression of several genes potentially affecting bone remodeling, including *Tnfsf11*. Interestingly however, and potentially explaining the absence of a pro-osteoclastogenic effect of IL-33, we failed to detect sRankl in the medium of osteoblasts after treatment with IL-33. In contrast, in the context of analyzing a mouse model with mucolipidosis-II, we found that sRankl was detectable in the medium of primary osteoblasts following treatment with PTH for 6 h [11]. This apparent inconsistency led us to directly compare the effects of IL-33 and PTH on *Tnfsf11* expression and sRankl release.

Here we report that PTH and IL-33 induce *Tnfsf11* expression to a similar extent, whereas sRankl release into the medium is specifically induced by PTH. Since this unexpected observation suggested that understanding this remarkable difference could lead to the identification of Rankl-specific endopeptidases, we performed additional experiments, including genome-wide expression analysis of primary osteoblasts after short-term treatment with PTH. Most importantly however, by analyzing the response of primary osteoblasts towards several *Tnfsf11*-inducing molecules, we identified a unique property of PTH, which was the only molecule causing sRankl release after short-term administration. Taken together, our data provide novel insights into the regulation of bone remodeling by PTH and the ectodomain shedding of Rankl.

2. Materials and methods

2.1. Primary murine osteoblasts

Primary osteoblasts were obtained from the calvariae of 5 days old male C57BL/6 wildtype mice. The key finding regarding the differential effect of IL-33 and PTH on Rankl processing was also confirmed in cell cultures obtained from female mice (Fig. S2). Cells were isolated by sequential digestion with collagenase/dispase and plated in α-MEM (Sigma-Aldrich) including 10% fetal bovine serum at a density of 130 cells per mm^2. After three days the cells were cultured in medium supplemented with ascorbic acid (50 μg/ml, Sigma-Aldrich) and β-glycerophosphate (10 mM, Sigma-Aldrich) for 10 days. Treatment with IL-33 (100 ng/ml, R&D Systems), hPTH [1–34] (10 nM, Bachem Inc.), IL-1β (100 ng/ml, Peprotech Inc.), IL-6 (100 ng/ml, Peprotech Inc.), soluble IL-6 receptor (100 ng/ml, R&D Systems), IL-17 (10 ng/ml, Peprotech Inc.), 1,25$(OH)_2$-vitamin-D3 (10 nM, Sigma-Aldrich), TNFα (10 ng/ml, Peprotech Inc.), IFN-γ (100 IU/ml, Peprotech Inc.), all-trans retinoic acid (1 μM, Sigma-Aldrich), prostaglandin-E2 (10 nM, Sigma Aldrich) or isoproterenol (10 μM, Sigma Aldrich) was performed in serum-free medium for 6 h after serum starvation of the cultures over night. To demonstrate that the release of sRankl induced by PTH was dependent on a metalloprotease activity, we added marimastat (Sigma-Aldrich) at a final concentration of 10 μM together with PTH. While RNA was isolated for expression analysis, the medium was collected for sRankl detection.

2.2. Human osteosarcoma cell lines

Human osteosarcoma cell lines (SaOS-2 and U2-OS) were obtained from ATCC (#HTB-85 and #HTB-96). The cells were plated at a density of 210 cells per mm^2 and differentiated for 5 or 10 days using commercially available osteoblast mineralization medium (PromoCell #C27020). Treatment with hPTH [1–34] was performed in serum-free medium for 6 h after serum starvation of the cultures over night. While RNA was isolated for expression analysis, the medium was collected for sRANKL detection by ELISA.

2.3. Expression analysis

RNA was isolated using the RNeasyMini kit (Qiagen), and DNase digestion was performed according to manufacturer's instructions. Concentration and quality of RNA were measured using a NanoDrop ND-1000 system (NanoDrop Technology). For qRT-PCR expression analysis, 1 μg of RNA was reversed transcribed using Verso cDNA Synthesis Kit (Thermo Fisher Scientific) according to manufacturer's instructions. The quantitative expression analysis was performed using a StepOnePlus system and predesigned TaqMan gene expression assays (Applied Biosystems). *Gapdh* expression was used as an internal control. Relative quantification was performed according to the $\Delta\Delta C_T$ method, and results were expressed in the linear form using the formula $2^{-\Delta\Delta CT}$. For genome-wide expression analysis, 5 μg of RNA were used for first strand cDNA synthesis. Synthesis of biotinylated cRNA was carried out using the IVT Labeling Kit (Affymetrix). For Gene Chip hybridization, the fragmented cRNA was incubated in hybridization solution at 45 °C for 16 h, before the Gene Chips (Affymetrix MG 430 2.0) were washed using the Affymetrix Fluidics Station 450. Microarrays were scanned with the Affymetrix Gene Chip Scanner 7G, and the signals were processed using GCOS (Affymetrix). To compare samples, the trimmed mean signal of each array was scaled to a target intensity of 300. Absolute and comparison analysis was performed using the Affymetrix MAS algorithm. Annotations were further analyzed with interactive query analysis at www.affymetrix.com.

2.4. sRankl detection

To quantify medium or serum concentrations of sRankl we utilized commercially available ELISA systems (R&D Systems, #MTR00 and #DY626 for mouse and human Rankl, respectively) following the manufacturer's instructions. For Western Blotting 10 μl medium or 20 μg of cell lysate was subjected to SDS-PAGE and then transferred to PVDF membranes (GE Healthcare) using 25 mM Tris-HCl (incl. 192 mM Glycin, 20% Methanol, pH 8.3) as a transfer buffer. After blocking the membranes with 5% skim milk powder in 1× TBST they were incubated with antibodies against murine Rankl (R&D Systems, AF462) and secondary antibodies (Polyclonal Rabbit Anti-Goat, Dako, #0449). Bound antibodies were detecting by chemilumiscence after incubation with luminol (500 mM, Sigma-Aldrich) and p-coumaric acid (80 mM, Sigma-Aldrich) in buffer (0,1 M Tris pH 8.5).

2.5. Osteoclastogenesis assays

Osteoclast precursor cells were isolated from the bone marrow of 12 weeks old mice and plated in α-MEM (Sigma-Aldrich) including 10% fetal bovine serum at a density of 2600 cells per mm^2. After one day the cells were cultured in medium supplemented either with 1,25$(OH)_2$-vitamin-D3 or hPTH [1–34]. On day 7 the osteoclast number was determined, RNA was isolated for expression analysis and medium was collected for sRankl detection.