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RARγ is a negative regulator of osteoclastogenesis

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ABSTRACT

Vitamin A is known to influence post-natal bone content, with excess intake being associated with reduced bone mineral density and increased fracture risk. Despite this, the roles retinoids play in regulating osteoclastogenesis, particularly *in vivo*, remain unresolved. This study therefore aimed to determine the effect of loss of retinoic acid receptors (RAR)α or RARγ on bone mass (analyzed by histomorphometry and dual-energy X-ray absorptiometry) and osteoclastogenesis in mice *in vivo*. RARγ null mice had significantly less trabecular bone at 8 weeks of age compared to wildtype littermates. In contrast, no change in trabecular bone mass was detected in RARα null mice at this age. Further histomorphometric analysis revealed a significantly greater osteoclast surface in bones from 8-week-old RARγ null male mice. This *in vivo* effect was cell lineage autonomous, and was associated with increased osteoclastogenesis *in vitro* from hematopoietic cells obtained from 8-week-old RARγ null male mice. The use of highly selective agonists in RANKL-induced osteoclast differentiation of wild type mouse whole bone marrow cells and RAW264.7 cells *in vitro* showed a stronger inhibitory effect of RARγ than RARα agonists, suggesting that RARγ is a more potent inhibitor of osteoclastogenesis. Furthermore, NFAT activation was also more strongly inhibited by RARγ than RARα agonists. While RARα and RARγ antagonists did not significantly affect osteoclast numbers *in vitro*, larger osteoclasts were observed in cultures stimulated with the antagonists, suggesting increased osteoclast fusion. Further investigation into the effect of retinoids *in vivo* revealed that oral administration of 5 mg/kg/day ATRA for 10 days protected against bone loss induced by granulocyte colony-stimulating factor (G-CSF) by inhibiting the pro-osteoclastogenic action of G-CSF. Collectively, our data indicates a physiological role for RARγ as a negative regulator of osteoclastogenesis *in vivo* and *in vitro*, and reveals distinct influences of RARα and RARγ in bone structure regulation.

1. Introduction

Skeletal development and maintenance of adult bone structure is a tightly regulated process relying on balanced actions of bone-resorbing osteoclasts and bone-forming osteoblasts [1]. When this balance is perturbed, clinical outcomes of osteopetrosis or osteoporosis can occur. High doses of the steroid hormone vitamin A (retinol) have been linked with increased bone resorption and bone loss. A recent meta-analysis of prospective clinical studies found that high intake of vitamin A as well as either low or high serum retinol levels are associated with greater risk of hip fracture [2]. Although the roles of retinoids in embryonic limb

Abbreviations: RAR, retinoic acid receptor; RXR, retinoid X receptor; ATRA, all-*trans* retinoic acid; BMM, bone marrow macrophages; G-CSF, granulocyte colony stimulating factor; RANKL, receptor activator of nuclear factor kappa-B; NFAT, nuclear factor of activated T cells; WBM, whole bone marrow; BMD, bone mineral density; DXA, dual X-ray absorptiometry; TNFα, tumour necrosis factorα.

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bud formation and skeletal patterning are well-defined, information on the effects of retinoids in post-natal skeletal development is very limited [3].

Retinoid signalling occurs *via* nuclear retinoic acid receptor (RAR) and retinoid X receptor (RXR) heterodimers. There are three subtypes each of RAR and RXR: α,β and γ. RARs are expressed in cells of the osteoclast and osteoblast lineage [4–7]. There is conflicting evidence regarding their direct roles in osteoblasts and osteoclasts, with numerous studies reporting both positive and negative effects on differentiation and activity. For example, all-*trans* retinoic acid (ATRA), the biological ligand for all three RARs, has been reported to both inhibit [8] and promote [9,10] differentiation and activity of osteoblasts. However, each study used different doses, cell lines and experimental techniques, making it difficult to discern the direct impact of retinoid exposure on osteoblasts. ATRA has been reported to stimulate osteoclast resorption in *ex vivo* bone cultures [11] but also to inhibit receptor activator of nuclear factor kappa-B (RANKL)-mediated osteoclastogenesis in osteoclast precursor cultures free of osteoblasts, specifically RAW264.7 cells, bone marrow macrophages (BMMs), bone marrow cells and spleen cells [12]. Thus, the direct effects or retinoids on individual cell types of the skeleton, and whether retinoids physiologically affect bone cell differentiation or activity remains unclear.

We previously reported that mice null for *Rarg* develop a myeloproliferative syndrome dependent on the bone marrow microenvironment [13]. In that study we noted that *Rarg* null mice had less trabecular bone, suggesting a role for RARγ in regulating bone mass. *Rarg*$^{-/-}$ mice also have an elevated frequency of axial skeleton defects [14,15], which occur at a much lower frequency in *Rara*$^{-/-}$ and *Rarb*$^{-/-}$ mice [15,16], indicating a role for RARs in skeletogenesis. The abnormalities exhibited by *Rara*$^{-/-}$ and *Rarg*$^{-/-}$ mice are more severe and result in early lethality [15], whereas *Rarb*$^{-/-}$ mice appear normal and have a full life expectancy [17]. Furthermore, previous studies have reported a role for RARα in regulating osteoclastogenesis *in vitro* [12]. Thus we investigated the roles of RARα and RARγ in post-natal bone *in vivo*.

2. Materials and methods

2.1. Mice

Rara [18] and *Rarg* [14] null mice were the kind gift of Professor Pierre Chambon. For ATRA/G-CSF studies, male C57BL/6 mice were obtained from Animal Resources Centre, Perth, WA, Australia. All experiments performed were approved by the Peter MacCallum Cancer Centre Animal Experimentation Ethics Committee and were conducted in strict compliance to the regulatory standards of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.2. Retinoid and G-CSF treatments

IRX5183 (RARα agonist), IXR4647 (RARγ agonist), IRX6996 (RARα antagonist), IRX5099 (RARγ antagonist), IRX4310 (RAR pan-antagonist) were kindly supplied by R. Chandraratna (Io Therapeutics, California) [19]. For *in vitro* studies, IRX ligands and ATRA (Sigma, St. Louis, MO) were dissolved in DMSO and diluted at 1/1000 for final concentrations in media. For *in vivo* studies, ATRA or an equal volume of DMSO was diluted in peanut oil and gavage fed to mice at 5 mg/kg/day for 10 days as previously described [20]. Recombinant human G-CSF (Filgrastim/Neupogen, Amgen, Thousand Oaks, CA) was diluted in sterile saline and s.c. injected at 125 μg/kg twice daily for 4 days from day 7–10 of ATRA treatment [20]. An equal volume of saline was injected in control mice.

2.3. Bone densitometry and histomorphometry

Mice were anesthetised with tribromoethanol (600 mg/kg) and bone densitometry was performed by dual-energy X-ray absorptiometry (DXA) on a Lunar PIXImus Densitometer (GE Medical Systems). Tibiae collected for histomorphometry were fixed in 4% paraformaldeyhyde and embedded in methylmethacrylate [21]. 5 μm sections were stained with toluidine blue or Xylenol Orange [22]. Histomorphometric analysis of undecalcified of trabecular bone of the secondary spongiosa of the proximal tibia and cortical bone of the antero-fibular tibial mid-diaphysis was performed using the Osteomeasure system (OsteoMetrics Inc., Decatur, GA) as previously described [21,23].

2.4. Quantitation of osteoclast progenitor cells

CSF-1-responsive agar colony-forming unit (CFU) assays were performed as previously described [24]. Osteoclast assays were performed as previously described [25]. Immunophenotypical analysis of osteoclast progenitors was performed in spleen and bone marrow (BM) preparations using FITC-conjugated CD11b (BD Pharmingen, San Diego, California) and PE-conjugated F4/80 (Caltag, South San Francisco, CA) as previously described [26]. Isotype-matched antibodies were used to determine background staining. Cells were analyzed on a FACScan (Becton Dickinson, San Jose, CA).

2.5. RNA extraction and quantitative RT-PCR

Spleens and whole marrow flushed from femurs was used for gene expression analysis. mRNA extraction, synthesis of cDNA and qRT-PCR were performed as previously described [27]. All primers used in these studies are given in Supplemental Table 1.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.jsbmb.2015.03.005.

2.6. Osteoclast cultures with RAR ligands

Mouse WBM, spleen or RAW264.7 cells were cultured as previously described [28]. Cells were plated at 10,000 RAW264.7, 500,000 spleen or 100,000 WBM cells/well in 6 mm diameter tissue culture wells. Cells were stimulated to differentiate for 6 days with 50 ng/ml of RANKL (plus 25 ng/mg M-CSF for WBM) in the presence of 10 nM, 100 nM or 1 μM RAR ligands or an equal volume of DMSO. Osteoclasts were identified by tartrate-resistant acid phosphatase (TRAP) histochemical staining and >2 nuclei. NFAT reporter assay was performed using RAW264.7 cells transfected with a GL4.30 (luc2P/NFAT-RE/Hygro) reporter construct, as described previously [28].

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0. Results are expressed as mean ± SD for n samples. Data were analyzed using an unpaired t-test or one-way ANOVA and Tukey's multiple comparisons test. Data is considered statistically significant if $p < 0.05$.

3. Results

3.1. RARγ null mice have reduced trabecular bone mass

DXA analysis of *Rarg*$^{-/-}$ mice revealed significantly lower (~10%) humeral BMD compared to wild type controls. This was noted in both female and male 8-week-old *Rarg*$^{-/-}$ mice compared to wildtype (Fig. 1A and B). Male *Rarg*$^{-/-}$ mice also showed