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Erythropoietin in bone – Controversies and consensus

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ABSTRACT

Erythropoietin (Epo) is the main hormone that regulates the production of red blood cells (hematopoiesis), by stimulating their progenitors. Beyond this vital function, several emerging roles have been noted for Epo in other tissues, including neurons, heart and retina. The skeletal system is also affected by Epo, however, its actions on bone are, as yet, controversial. Here, we review the seemingly contradicting evidence regarding Epo effects on bone remodeling. We also discuss the evidence pointing to a direct *versus* indirect effect of Epo on the osteoblastic and osteoclastic cell lineages. The current controversy may derive from a context-dependent mode of action of Epo, namely opposite skeletal actions during bone regeneration and steady-state bone remodeling. Differences in conclusions from the published *in-vitro* studies may thus relate to the different experimental conditions. Taken together, these studies indicate a complexity of Epo functions in bone cells.

1. Introduction

Erythropoietin (Epo) is a hormone that stimulates red blood cell differentiation. It acts *via* binding to its receptor (Epo-R) on erythroid progenitor cells within the bone marrow. Epo-R downstream pathways are mediated by Janus kinase 2 (JAK2), STAT3 and STAT5 and signal through phosphatidylinositol-3 kinase (PI3K)/Akt, MAP kinase, and protein kinase C [1].

The primary site of Epo production resides in the fetal liver and adult kidney, where *Epo* gene expression occurs mainly under the control of an oxygen-sensing, hypoxia-inducible factor-dependent mechanism [2]. The expression of Epo-R in non-erythroid tissues such as the brain, retina, heart, kidney, smooth muscle, and vascular endothelium, as well as macrophages and dendrocytes, has been associated with the discovery of novel biological functions of endogenous Epo signaling, unrelated to hematopoiesis [3–9]. However, the expression and functional relevance of Epo-R in non-erythroid cells are still a matter of debate [10,11]. It is expected that the recent development of a more specific and sensitive antibody against human Epo-R will contribute to resolving these questions [12,13].

Several roles have been attributed to Epo in bone biology over the last two decades; yet, the conclusions were controversial [14]. This review aims at describing the consensual findings and an attempt is made to resolve some of the current controversies related to Epo involvement in skeletal biology.

Osteoclasts are derived from the monocyte/macrophage lineage [15]. They differentiate in the proximity of bone surface and their main function is resorption of the mineralized matrix. Osteoclastogenesis is under the tight regulation of the TNF-related cytokine receptor activator of NFκB (RANK) ligand (RANKL) and colony-stimulating factor-1 (CSF-1, M-CSF). Activation of RANK, the receptor of RANKL, on the surface of monocyte/macrophage precursor cells induces expression of osteoclastic related genes, including those encoding tartrate-resistant acid phosphatase (TRAP), cathepsin K (CatK), calcitonin receptor and the $\alpha v\beta_3$-integrin, leading to osteoclast maturation and resorbing activity [16].

Osteoblasts arise from a common pluripotent mesenchymal stem cell (MSC) [17]. Bone Morphogenetic Proteins (BMPs) and

Abbreviations: ALP, Alkaline phosphatase; BFR, bone formation rate; BSP, Bone sialoprotein; BV/TV, bone volume/total volume, trabecular bone fraction; CTX, carboxy-terminal collagen crosslinks; Conn.D, connectivity density; rHuEPO, Erythropoietin (EPO)/recombinant human EPO; EPO-R, Erythropoietin receptor; FBS, fetal bovine serum; M-CSF, macrophage colony stimulating factor; M-CSF-R, CSF1-R, cFms, macrophage colony stimulating factor receptor; MSCs, mesenchymal stem cells; Tg6, mice over-expressing human EPO gene; μCT, microcomputed tomography; MAR, mineral apposition rate; MS/BS, mineralizing surface/bone surface; OCN, Osteocalcin; N.Oc/BS, osteoclast number/bone surface; OPG, osteoprotegerin; RANK, receptor activator of nuclear factor kappa B; RANKL, receptor activator of nuclear factor kappa B ligand; TRAP, tartrate-resistant acid phosphatase; Tb.N, trabecular number; WT, wild type.

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Wnt pathways are especially crucial for the early steps of osteoblastogenesis, where they promote the expression of osteogenic genes such as RUNX2, leading to their commitment toward an osteo/chondroprogenitor. RUNX2 has been shown to upregulate osteoblast-related genes such as *Col1α1*, Tissue Non-Specific Alkaline Phosphatase – TNSALP, Bone sialoprotein 2 (*BSP*) and Osteocalcin (OCN, a.k.a. *BGLAP*). The main role of osteoblasts is the deposition of bone matrix and mineralization, and they have also been implicated in extra-skeletal function such as energy metabolism and male fertility [16].

2. *In vitro* effects of Epo

2.1. Expression of Epo-R in bone cells

The expression of Epo-R in non-erythroid cells in the bone marrow was challenged by a study based on lineage tracing of Epo-R promoter activity, using the CRE-loc technology [11]. Based on that study, it was concluded that expression of Epo-R is essentially restricted to erythroid lineage cells. In contrast, other groups demonstrated Epo-R expression at the RNA level in macrophages, differentiating preosteoclasts and mature osteoblasts [18,19]. Moreover, specific Epo-R signaling and functional assays in response to Epo stimulation was shown in isolated cells from the osteoblastic and monocytic lineages [9,19–22]. A careful analysis of the data presented by Singbrant et al. reveals that the cell sorting in that study was limited to a small number of cell types and for some of them, only to an early differentiation stage [11]. Importantly, they did not show any data on mature osteoblasts and preosteoclasts/osteoclasts, where studies by others specifically found high Epo-R expression. There is therefore no contradiction between the aforementioned studies, and it can be concluded that in addition to erythroid cells, Epo-R is indeed expressed at specific developmental stages in both the osteoblastic and monocytic lineages. However, although the functional relevance of this expression has been repeatedly demonstrated *in vitro*, the physiologic and therapeutic roles of Epo-R in bone cells remain to be validated *in vivo*. This can be accomplished, for instance, by using animal models, such as conditional deletion of Epo-R in particular bone cells.

2.2. Actions of Epo signaling in osteoblastic cells

As mentioned above, Epo-R expression increases during osteoblast differentiation [19] and upon Epo administration to osteoblasts *in vitro* [23]. Epo was reported to induce osteogenic differentiation and mineralization in human and rodent bone marrow osteoblasts, as well as in the ST2 osteoblastic cell line cultured with Epo doses between 10 and 100 U/ml [21–24]. The osteogenic effect of Epo was shown to be mediated by the mTOR, JAK2 and PI3K signaling pathways [21,22]. These *in vitro* data lend support to the notion that osteoblasts express Epo-R and respond to Epo activation. However, the physiologic relevance of these findings is questioned, in view of the relatively high Epo doses necessary to trigger the osteogenic response. Whereas clinically, the basal plasma concentration of Epo ranges from 6 to 32 mU/ml [25], mineralization in osteoblast cultures was not stimulated by Epo at doses below 1–10 U/ml [19,21,23]. It would be interesting to test the response of osteoblasts to Epo at even lower doses, e.g. 1–100 mU/ml.

2.3. Epo signaling in osteoclastic cells

Studies by different groups, showed that Epo promoted *in vitro* osteoclast formation at doses ranging from 5 to 20 U/ml [18,19,22,24,26]. Signaling of Epo-R in preosteoclasts was mediated by JAK2 and PI3K, independently of MAPK [19].*In vitro* concentrations above 5 U/ml were at least 2 orders of magnitude above the physiologic range in humans [25]. Therefore, we have recently reexamined the effect of Epo on osteoclastogenesis at a dose of 10 mU/ml, using otherwise identical methodology as in Hiram-Bab et al. [19]. We found that osteoclast differentiation was stimulated even at these low, "physiological" concentrations (unpublished data, Fig. 1).

One report suggested that Epo administration during RANKL-induced osteoclastogenesis in primary osteoclast precursors, resulted in the formation of inactive osteoclasts [22]. Repeating this assay with a slightly different calcium-coated plate type [19], we found that osteoclastogenesis was associated with a similar significant increase in pit formation, and the resorbing activity per cell was not impaired by Epo. This was further supported by *in vivo* data showing an overall increase in the bone resorption marker TRAP5b, associated with bone resorption in Epo-overexpressing (Tg6), as well as in Epo-injected mice [19]. We also recorded increased serum levels of carboxy-terminal collagen cross-links (CTX), another marker of bone resorption (Mean ± SD: 59.1 ± 15.2 *versus* 30.9 ± 14.9 ng/ml in Tg6 *versus* WT female mice, respectively, $n = 7$ for each group, $p < 0.002$; unpublished data).

3. Epo effects on bone regeneration

Studies on the effects of Epo on bone were carried out *in vivo* mainly on models of bone regeneration, including mice, rabbits and pigs, as well as in clinical trials [26–34]. Holstein et al. [32] reported for the first time the beneficial effects of short-term Epo treatment in fracture repair. Although not all articles agree [35], the general effect of Epo in fracture healing includes increased callus volume and mineral content, accelerated fracture bridging and enhanced biomechanical properties [28–32,34].

Bone healing can occur in two main processes, namely primary/cortical or secondary/endochondral [36]. Primary cortical healing takes place when the stability of the fracture is absolute. Healing is characterized by cortical osteonal healing, leading to direct fusion of the fracture ends by cortical bone. Secondary

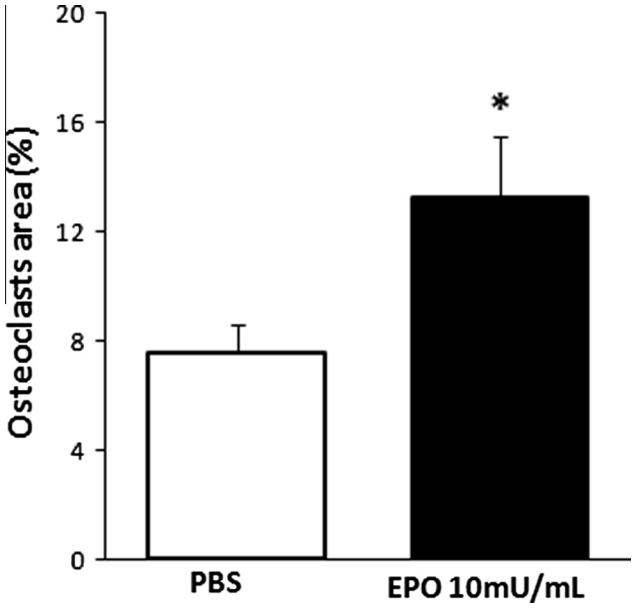

Fig. 1. Epo induces osteoclastogenesis at low dose. Bone marrow-derived macrophages were differentiated in osteoclastogenic medium for 5 days in the presence of Epo 10 mU/ml or PBS. Osteoclasts were stained and counted as previously described [18]. Graphs are mean ± SEM of three independent experiments. *, $p = 0.034$.