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Review article

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.

Contents

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,

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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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 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

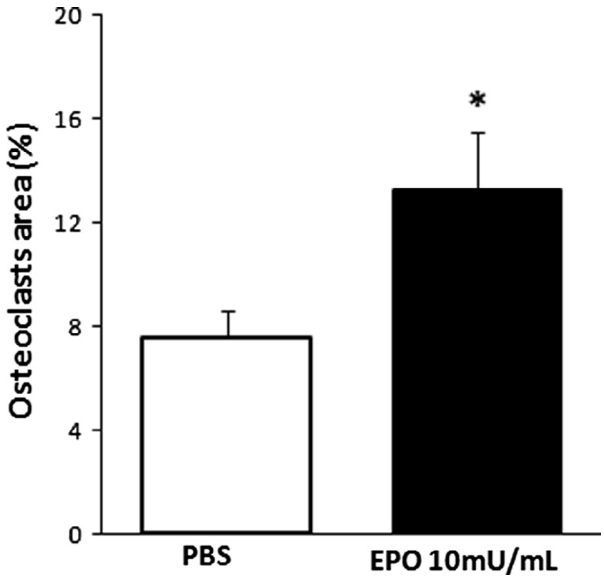

Fig. 1. Epo induces osteoclastogenesis at low dose. Bone marrow-derived macrophages were differentiated in osteoclastogenic medium for 5 days in the presence of Epo 10 mU/ml or PBS. Osteoclasts were stained and counted as previously described [18]. Graphs are mean ± SEM of three independent experiments.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

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 healing occurs in cases of non-rigid fixation and is mediated by the formation of a cartilaginous callus that rapidly forms, calcifies and remodels into woven and trabecular bone. Secondary healing is reminiscent of embryological bone development and includes both models of intramembranous and endochondral bone formation. In both primary and secondary bone healing, the early stages include an acute inflammatory response and activation of angiogenesis [36,37].

Interestingly, Epo's bone anabolic effect was reported on both models of primary and secondary healing. It is therefore likely to assume that the effect of Epo is not dependent on chondrocytes. This assumption is supported by a report showing no beneficial effect of Epo in a model of osteochondral defect [33]. In that study, an improvement in cartilage healing was evident only when Epo was administered in addition to bone marrow aspiration concentrate containing osteoblast progenitors.

Epo-induced osteogenesis in bone healing and regeneration models was repeatedly attributed to the direct stimulation of osteoblast proliferation and differentiation observed *in vitro*, as discussed above. In addition, Epo was found to act on other cells, leading to an indirect stimulation of osteoblasts [18]. Different studies suggested that such indirect effects were mediated by hematopoietic stem cells (HSC) and/or osteoclasts. Epo was suggested to enhance HSCs population [18,26] and to increase BMP2 expression in these cells [18], which in turn stimulates osteogenic differentiation of osteoblasts. The importance of this pathway is advocated by the fact that Epo administration had no additive effect over BMP2 alone [26], and that Epo's impact on bone regeneration was blunted in the absence of BMP2 [38]. These studies suggest that the osteogenic function of Epo is mediated, at least in part, by BMP2 expressed in HSCs.

Neighboring osteoclasts (and/or preosteoclasts) also appear to mediate the osteogenic stimulatory effect of Epo. Osteoclastic cells express EphrinB2, a coupling factor that positively regulates osteoblast differentiation by binding to its receptor EphB4 harbored on osteoblast precursors [39]. Epo was shown to upregulate the expression of EphrinB2 in osteoclasts and that of EphB4 receptor in both bone marrow stromal cells (osteoblast progenitors), as well as in the ST2 osteoblastic cell line [24]. The functional occurrence of this process, namely Epo stimulation of osteoblast differentiation *via* increased expression of EphrinB2 in osteoclasts, remains to be demonstrated by using relevant experimental strategies, such as co-cultures, or conditional knockout models.

In addition to the direct activation of osteoblasts, or their neighboring HSCs and osteoclasts, the beneficial role of Epo could be attributed also to early effects on the post-traumatic inflammatory and angiogenic responses. Epo has been associated with reduced inflammation in many conditions, such as secondary burn progression [40], intestinal inflammation [41], hepatic injury [42], and suppression of TLR2/NF-κB-mediated inflammation [31,42].

Simultaneously to endochondral ossification, new blood vessels invade the callus. Neoangiogenesis plays a critical role in the process of osteogenesis. Indeed, in most of the studied models of bone repair, Epo was associated with an increased number of blood vessels [26,28–31,34]. One of the suggested mechanisms is that Epo increases the number of endothelial progenitor cells in the peripheral blood, and their recruitment to the site of injury [31]. Epo was also shown to increase expression of VEGF [29], an important angiogenic factor. Interestingly, Sun et al. [26] showed that Epo-induced angiogenesis was largely blunted when osteoclastogenesis was blocked by bisphosphonates or osteoprotegerin (OPG) in a metatarsal organ culture assay. It thus appears that osteoclastogenesis is essential for the angiogenesis process. This is also in line with the reported increase in osteoclast number in Epo-treated mice [24,26].

In all these models of fracture healing and regeneration, bone trauma triggers a complex reaction involving a variety of cytokines and cell types. Notably, Epo seems to affect a series of different steps during this process, such as acute inflammatory response, callus formation and mineralization, and angiogenesis. These processes involve the mobilization of a variety of cell types, including osteoclasts, osteoblasts, endothelial and blood cells, and their respective progenitors. During the surgical phase of these experimental models, blood loss and bone trauma often result in a significant increase in Epo production, in addition to the exogenously administered Epo. In line with the Epo dosage required to induce osteogenesis *in vitro*, the bone anabolic action of Epo is likely to be restricted to its very high circulating concentrations.

4. Regulation of bone homeostasis by Epo

Physiologic bone remodeling is a life lasting process, where mature bone tissue is removed (bone resorption) and replaced with new bone tissue (bone formation). These processes control the reshaping, or the replacement of bone following microdamage that occurs during normal activity. Therefore, an imbalance in the regulation of bone resorption and formation, results in changes in bone mineral density. In contrast with bone remodeling, the traumatic models of bone healing or regeneration are accompanied with noticeable inflammatory reaction, neoangiogenesis, and hypoxia. To study the skeletal effect of Epo during steady state bone remodeling, independently of these processes, a few groups have used atraumatic animal models.

We have recently studied the role of Epo during adult bone remodeling. We used several mouse models, namely Epo overexpressing mice (Tg6), intermittent injections of moderate and high Epo doses, and continuous administration of moderate doses [19]. In Tg6 mice, the increase in Epo levels is similar to the stimulation of Epo at high altitude [19,43]. This model is also relevant for the pathophysiologic increase of endogenous Epo levels observed after bleeding/repeated phlebotomies or bone injuries, without the confounding direct effect of hypoxia on bone cells [44,45]. On the other hand, the Epo-injection model is relevant for therapeutic administration of exogenous Epo [46]. Noteworthy, recombinant human Epo is widely prescribed for treating anemia in patients with end-stage renal disease, and for years it has been the highest-expenditure drug by Medicare in the USA.

In all the atraumatic adult mouse models reported by us and others, high serum levels of Epo resulted in a lower trabecular bone

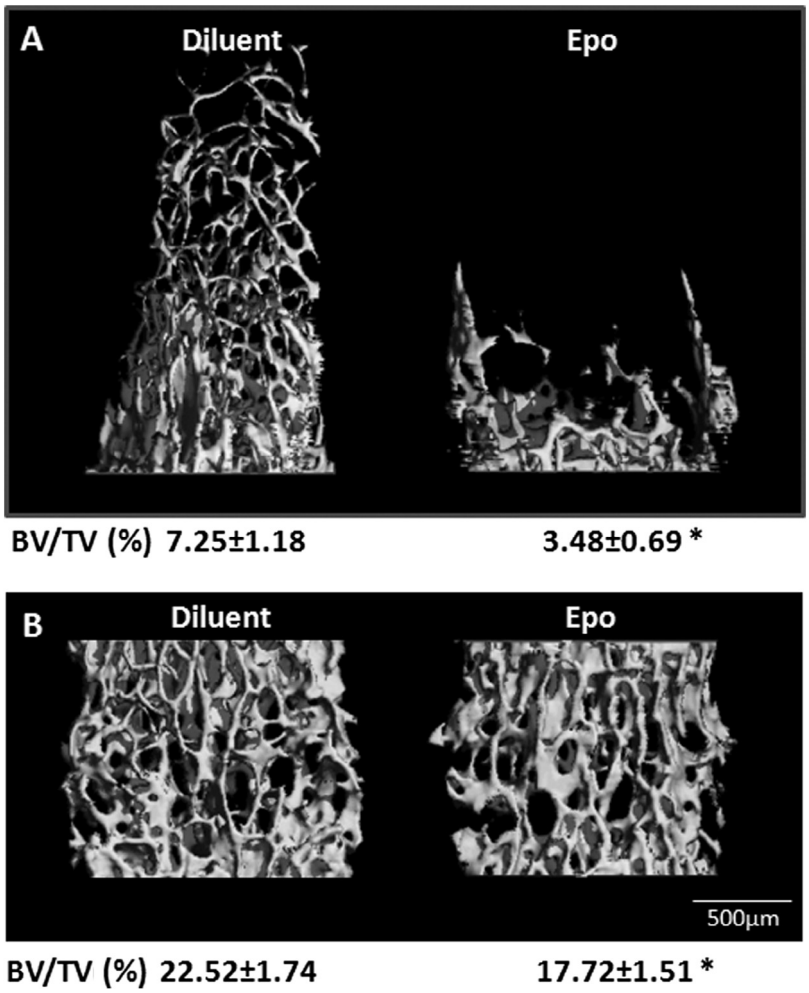

Fig. 2. Epo induces bone loss in growing mice. 3D images and trabecular bone fraction (BV/TV) in femurs (A) and L3 vertebra (B) of 8w C57Bl/6J mice treated between the age of 4 week and 8 week with Epo (120U, 3 times/week for 4 weeks) or diluent. $n = 8$ for each group. *, $p < 0.05$.

mass (bone volume fraction), mainly due to lower trabecular number [11,19,47]. This low bone mass was associated with lower bone formation and higher bone resorption levels, as compared to controls [19]. In that regard, Singbrant et al. [11] agreed with the stimulatory effect of Epo on osteoclastogenesis, but they concluded that Epo also increased bone formation based on the observed increase in osteoblast number. However, a careful analysis of the presented data reveals that in Epo-treated mice, the single labeled calcein, which is a surrogate of osteoblasts' activity, was significantly decreased [11], in line with our reported inhibition of bone formation by Epo [19]. The attenuation of bone formation was further supported by the significant decrease in serum levels of osteocalcin observed in the Tg6 mice [19].

In contrast to adult animals, one study on growing mice (newborn and 4–6 weeks of age) treated for 4 weeks, showed that Epo increased bone mass in an atraumatic model [18]. Using the same experimental conditions in growing female mice (4 weeks of age) treated for 4 weeks, we found that the effect of Epo was very similar to that observed in adult animals, in both the femur and vertebrae (Fig. 2, unpublished data). This discrepancy between our findings and the earlier report on a similar experimental design [18] might be attributed to genetic drift of the mouse strain, environmental conditions and/or slight differences in the growth rate of the animals.

It is interesting to note that in newborn mice (treated from birth to day 28), bone growth is strongly associated with hypoxia and angiogenesis, similarly to traumatic bone healing [48]. The important stimulatory role of Epo on these two processes involved during bone growth and other traumatic bone models appears, therefore, to supersede the inhibitory effect of Epo on steady-state bone formation. It is likely to assume that the opposite action of Epo in the traumatic/skeletal growth models [18] *versus* adult bone remodeling [11,19] is attributed to the fact that hypoxia and angiogenesis play dominant roles in the former, but not in the latter process.

5. Conclusions

In adult bone homeostasis, bone loss is driven mainly by the inhibition of bone formation and stimulation of bone resorption. Contradicting reports on the effect of Epo on bone during skeletal growth and bone healing may be attributed to the involvement of Epo-stimulated angiogenesis and response to hypoxia. *In vitro*, the stimulatory effect of Epo on osteoclastogenesis is widely accepted. Controversy remains as to the stimulation of bone resorbing activity. Regarding direct effect of Epo-R signaling in osteoblasts, the discrepancy between stimulatory (*in vitro*) and inhibitory (*in vivo*) actions of Epo may be due either to the involvement of other cell types and/or to the dosage. Based on the physiological concentrations of serum Epo, one may assume that concentrations below 100 mU/ml are more clinically relevant than the doses that induce an osteogenic response *in vitro*. As such, further studies are warranted to examine the direct effect of Epo on osteoblasts at low concentrations. This discrepancy may also result from the involvement of other cell types *in vivo*. As an example,

Epo may act on pre-osteoclasts to modulate the expression of osteoclasts-to-osteoblasts coupling signals [49].

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