Published in final edited form as:

Growth Factors. 2012 February ; 30(1): 22–28. doi:10.3109/08977194.2011.637034.

Effects of Erythropoietin on the Bone Microenvironment

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Abstract

It has well been established that blood and bone share a unique, regulatory relationship with one another, though the specifics of this relationship still remain unanswered. Erythropoietin (Epo) is known primarily for its role as a hematopoietic hormone. However, after the discovery of Epo receptor (Epo-R) outside the hematopoietic tissues, Epo has been avidly studied for its possible non-hematopoietic effects. It has been proposed that Epo interacts with bone both directly, by activating bone marrow stromal cells, and indirectly, through signaling pathways on hematopoietic stem cells (HSCs). Yet the role of Epo in regulating skeletal maintenance and regeneration remains controversial. Here we review the current state of knowledge pertaining to the effects of Epo on the skeleton.

Key Terms

Erythropoietin; Osteopoiesis; Osteogenesis; Bone Microenvironment

Introduction

Bone is a dynamic organ that maintains a diverse role within the body. The skeleton serves as the basis for locomotion, as a large mineral storage, and houses the hematopoietic organ. Within the marrow medullary cavity, the bone marrow microenvironment is a diverse cellular population including hematopoietic stem cells (HSCs), hematopoietic progenitor cells (HPCs), mesenchymal stem cells (MSCs), and mesenchymal progenitor cells (MPCs). The stromal compartment also contains cellular populations including fibroblasts, macrophages, adipocytes, osteoblasts, osteoclasts, reticular cells and endothelial cells (Anjos-Afonso and Bonnet 2007).

HSCs are found within an area of the marrow termed the ‘stem cell niche.’ The stem cell niche plays an important role in hematopoietic tissue maintenance, repair, and generation. It also provides protection for adult HSCs that are in a largely quiescent state, cycling very slowly, until they are called upon to respond to tissue injury. When tissue injury occurs, the niche communicates to the stem cells to either proliferate with self-renewal divisions or differentiate into mature blood cells. Emerging evidence suggests that as part of the stem

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Declarations of Interest

This study was supported by grants from the Department of Defense (PC073952) and National Institutes of Health (DK082481, DE020721, 1RC1DE020721, CA141426 and CA093900).

cell niche are osteoblasts, endothelial cells, adipocytes, reticular cells and MSCs (Dexter and Spooner 1987; Lemischka 1997; Moore, Pytowski et al. 1997). Like hematopoietic stem cells, MSCs are capable of self-renewal and are able to differentiate into multiple lineages including bone, adipocytes, cartilage, and hematopoietic-supporting cells (Taichman 2005).

Osteoblasts are chiefly responsible for the secretion of a mineralized matrix that serves as the basis of skeletal locomotion development. Osteoblasts are derived from pluripotent MSCs under the action of several bone morphogenic factors including the BMP family (IGFs and TGF- β) which activate specific transcription factors (Runx2, osterix AFT-4) necessary for osteoblastic growth (Taichman 2005). Osteoblasts are also influenced by many mechanical, local, and systemic stimuli, which serve to maintain skeletal homeostasis and bone remodeling. While osteoblasts are known to be a part of the stromal cell support system within the marrow, osteoblasts and their precursors have been shown to play a vital role in establishing the HSC niche (Taichman and Emerson 1994; Calvi, Adams et al. 2003; Arai, Hirao et al. 2004; Nilsson, Johnston et al. 2005; Taichman 2005; Adams, Chabner et al. 2006). This role was first reported in tissue culture studies (Taichman and Emerson 1994; Taichman, Reilly et al. 1996). Later these observations were confirmed in mouse transgenic models (Calvi, Adams et al. 2003; Zhang, Niu et al. 2003). A number of molecules have been implicated in the niche supportive role that osteoblasts play including N-cadherin (Zhang, Niu et al. 2003) jagged-1 (Calvi, Adams et al. 2003; Zhang, Niu et al. 2003), and VCAM-1 (Rose, Cardarelli et al. 2000) along with soluble and cell-surface associated cytokines and growth factors, which regulate HSC functions. Moreover, these interactions are modulated by mechanical, systemic hormones (e.g., PTH, Growth hormone) and local signals (e.g., BMPs, Ang-1) (Calvi, Adams et al. 2003; Zhang, Niu et al. 2003; Arai, Hirao et al. 2004).

Osteoblast function is intricately balanced with that of osteoclasts. Osteoclasts are large multinucleated cells of hematopoietic origin, which are responsible for bone resorption. Osteoclast and osteoblast interactions are crucial for the activation and coordination of resorption and formation which when balanced, maintains bone tissue in a homeostatic state. When osteoblast function falls behind the resorption activity of osteoclasts, osteopenia will result. Conversely, when osteoblastic activity is out of step with osteoclastic bone resorption, osteopetrosis occurs. These interactions are made possible by messages predominantly coordinated by the RANK/RANKL axis (Theoleyre, Wittrant et al. 2004). Soluble or cell associated RANKL expressed on the surface of osteoblasts is able to bind and activate osteoclasts by signaling through the RANK receptor. Osteoprotegerin, a soluble RANK decoy receptor, is able to mitigate the effects of RANKL to regulate the RANKL activation of osteoclasts (Theoleyre, Wittrant et al. 2004)

Erythropoietin

Erythropoietin (Epo) is most recognized for its function as a hematopoietic hormone secreted from the kidneys and liver in adult mammals in response to hypoxia by activating HIF-1 α (Tan, Eckardt et al. 1991; Brines, Grasso et al. 2004). In reaction to low oxygen levels, Epo is released into the peripheral circulation. Epo binds to a preformed homodimer transmembrane receptor (Epo-R) resulting in downstream phosphorylation of the Janus kinase (**JAK**)/signal transducer and activator of transcription (**STAT**) signal transduction cascades to induce proliferation and differentiation of hematopoietic precursors, thus expanding the production of erythrocytes (Brines, Grasso et al. 2004; Farrell and Lee 2004). Recent data shows that Epo-R physically interacts with the common beta receptor (β cR or CD131 or GP130), which is a shared subunit with granulocyte-macrophage colony stimulating factor, and the IL-3 and IL-5 receptors (Brines, Grasso et al. 2004). Data also shows that in β cR knocked out animals, normal erythropoiesis maturation occurs (Brines,

Grasso et al. 2004), suggesting that Epo binding to β cR is not required for erythropoiesis but involved in other tissue-protective mechanisms particularly in non-hematopoietic tissues. Thus, Epo-R and β cR binding may comprise a tissue-protective heteroreceptor for Epo's bone forming role (Brines, Grasso et al. 2004).

Epo receptors (Epo-Rs) have been reported to be present outside of hematopoietic tissues, suggesting that Epo plays a role in non-hematopoietic tissues as well (Mennini, De Paola et al. 2006). Epo-R mRNAs have been reported in the brain, testes, placenta, heart, lungs, bone marrow, and spleen (Tan, Eckardt et al. 1991; Fandrey and Bunn 1993; Agnello, Bigini et al. 2002; Brines and Cerami 2006). It has also been proposed that Epo expression itself is expressed in tissues outside of the kidney where it is regulated in a way that is specific to the tissue in which it resides and therefore Epo may have specific functions for different tissues (Haroon, Amin et al. 2003). For example, Epo and Epo-R play a role in embryonic and adult neural development and function (Tsai, Ohab et al. 2006). Tsai et al shows that mouse embryos lacking Epo and Epo-R develop incomplete or defected neural tubes (Tsai, Ohab et al. 2006). This same group also demonstrates Epo-R's importance in the movement of new neuroblasts to injured tissues (Tsai, Ohab et al. 2006). However, when exposed to stroke, the infarct sizes in control mice did not vary significantly to those of Epo-R knockdown mice, suggesting multiple motives for Epo's neuroprotective mechanisms (Tsai, Ohab et al. 2006). Epo has also demonstrated significant promise for tissue protection in the cardiovascular system. Multiple studies have demonstrated the potential Epo has as a treatment for myocardial infarction (Cai, Manalo et al. 2003; Calvillo, Latini et al. 2003; Moon, Krawczyk et al. 2003; Parsa, Matsumoto et al. 2003; Wright, Hanlon et al. 2004). After ischemia-reperfusion injury, Epo has been shown to aid in the survival of cardiomyocytes as well as the remodeling of the left ventricle (Tada, Kagaya et al. 2006). Low levels of Epo and Epo-R resulted in pressure overload-induced cardiac dysfunction, and ultimately, reduced survival (Asaumi, Kagaya et al. 2007). It should be noted that high doses of Epo were required in order to observe its tissue protective effects as compared to hematopoietic effects (Anagnostou, Liu et al. 1994; Ammarguella, Gogusev et al. 1996; Brines and Cerami 2005).

The differential role of Epo on different organs illustrates the tissue-specific functions of Epo (Haroon, Amin et al. 2003). Moreover, Epo has shown to possess tissue-protective effects by preventing vascular spasm, apoptosis and regulating inflammatory responses (Erbayraktar, Grasso et al. 2003; Brines, Patel et al. 2008). Interestingly, when there are chemical or amino acid modifications to the Epo binding domains, the ability of Epo/Epo-R binding was diminished (Coleman, Westenfelder et al. 2006; Erbayraktar, de Lanerolle et al. 2006; Fantacci, Bianciardi et al. 2006). Yet following deletion of Epo-R binding abolishes the erythropoietic effects, while retaining other tissue-protective properties, suggesting other Epo-Rs (Lacombe and Mayeux 1998; Cheung and Miller 2001; Brines, Grasso et al. 2004; Jelkmann 2004). It has been demonstrated that different intracellular pathways may be activated by specific Epo receptor conformations through the use of Epo receptor fusion proteins (Frank 2002). If this is the case, the signaling pathway to be activated may depend on the receptor conformation achieved (Frank 2002).

Influence of Epo on Bone

The finding that Epo has pleiotropic roles in non-hematopoietic tissues has prompted a search for a role of Epo in bone formation and homeostasis. The rationale for such a search is that crosstalk between hematopoietic and osteogenic cells is known to affect each other's function (Li and Li 2006; Wilson and Trumpp 2006; Yin and Li 2006). Therefore, the coupling of hematopoiesis with skeletal homeostasis through Epo signaling makes considerable sense. In fact, it has been shown that osteogenic activities *in vivo* are enhanced

following direct marrow ablation or trauma (Foldes, Naparstek et al. 1989; Gazit, Karmish et al. 1990; Bab, Gazit et al. 1992; Bab and Einhorn 1993; Bab 1995; Greenberg, Chorev et al. 1995). Moreover, after acute or chronic blood loss, mature blood elements need to be replaced, as illustrated in Figure 1B. In order for this process to take place, the fatty marrow cavity must be expanded into hematopoietic marrow, resulting in bone resorption to create sufficient space, shown in Figure 1C. During blood loss, HSCs are stimulated. But, as seen in Figure 1A, these activities may also activate osteoprogenitor cells in the bone marrow niche (Lucas, Bab et al. 1997). Thus when animals sustain an acute blood loss (1–3% of body weight) or injury to the tibial bone marrow, a concomitant increase in mineral appositional rates, serum levels of an osteogenic growth peptide, and osteoblast numbers have been observed (Lucas, Bab et al. 1997).

As suggested, in murine models, it has been demonstrated that an acute bleed may trigger bone formation as well as hematopoiesis (Foldes, Naparstek et al. 1989; Gazit, Karmish et al. 1990; Bab, Gazit et al. 1992; Bab and Einhorn 1993; Greenberg, Chorev et al. 1995; Lucas, Bab et al. 1997; Brager, Patterson et al. 2000). For example, Ilizarov *et al.* revealed that the rate of bone regeneration in animals increases after an acute blood loss. (Ilizarov, Chepelenko et al. 1986). On the other hand, chronically bled animals have shown heightened bone resorption (Gurevitch, Khitrin et al. 2007). As discussed above, fatty marrow can be converted to hematopoietic marrow when mature blood elements need to be replaced, providing possible reason to why chronically bled animals show symptoms similar to those of osteoporosis (Gurevitch, Khitrin et al. 2007).

The idea of chronic bleeding may apply to postmenopausal women experiencing osteoporosis as well. It has been estimated that a woman will lose 70 mL of blood per month for nearly 35 years, giving a lifetime loss of menstrual blood of 30 L (Gurevitch, Prigozhina et al. 1999), thus menstrual bleeding may stimulate bone homeostasis, which is lost in postmenopausal women. Osteoporosis, however, is not limited to postmenopausal women. Instead, it can be seen as an adverse side effect of several diseases and medications. For example, osteoporosis is a common result of β -thalassemia, a blood disorder where the production rate of hemoglobin is reduced and regular blood transfusions are needed. Interestingly, transfused individuals have improved bone mineral densities (Yazigi, Maalouf et al. 2002).

Evidence that osteopoiesis can be stimulated directly by Epo is limited and the few reports available are somewhat contradicting. For example, Takenaka *et al.* studied how Epo may affect patients on hemodialysis, more specifically, their bone metabolism (Takenaka, Itaya et al. 2003). When exogenous Epo was delivered, serum levels of 1,25-dihydroxy-vitamin D, intact osteocalcin and bone alkaline phosphatase were increased but not intact parathyroid hormone (Takenaka, Itaya et al. 2003). These data provide evidence that improved formation of bone matrix for patients with anemia on hemodialysis can be associated with Epo (Takenaka, Itaya et al. 2003). Supporting this observation, Lee *et al.* has shown that Epo treatment can increase cortical thickness even though osteoclast number increased along the endosteal surfaces in the marrow (Lee, Fukunaga et al. 1991).

To better understand if Epo can improve bone healing, Holstein et al. injected mice before and after femoral fracture with Epo (5000 U/kg/day) for a total of 6 days (Holstein, Menger et al. 2007). After allowing two weeks of healing time, Epo-R expression was noted in hypertrophic chondrocytes within the callus (Holstein, Menger et al. 2007). As compared to vehicle controls, Epo-treated mice demonstrated increased torsional stiffness and callus density. However, the effects of the Epo treatment were undetectable 5 weeks after fracture (Holstein, Menger et al. 2007).

To follow up this study, Garcia *et al.* studied the effects of lower doses (500U/kg/day) of Epo over a longer period of time of (5 weeks) on the fracture healing of mice with small segmental defects. An increase in the bending stiffness and radiological density of segmental the defects were improved by the treatment (Garcia, Speidel et al. 2011) and were maintained for longer than the initial two-week period. Epo levels are also linked to decreased levels of inflammation, however, in order to stimulate the healing process, an inflammatory response is necessary (Cuzzocrea, Mazzon et al. 2004; Einhorn 2005; Zhu, Jin et al. 2009). The degree of inflammation needed to invoke healing remains unknown as it has been shown that both increased and decreased inflammatory responses can weaken fracture healing (Reikeras, Wang et al. 2007; Yamaguchi, Takada et al. 2009). New bone formation can be triggered by the inhibition of inflammatory cytokines following injury particularly NFkB levels, as it is crucial in regulating inflammatory mediator production (Garcia, Speidel et al. 2011). In fact, Epo significantly reduces NFkB expression (Garcia, Speidel et al. 2011). Therefore, NFkB targeting by Epo may promote fracture healing in the Epo-treated mice (Garcia, Speidel et al. 2011).

The observation of improved fracture healing after Epo treatment is not limited to the mouse-model. Mihmanli *et al.* studied the effects of human recombinant Epo (rHuEpo) on mandibular distraction osteogenesis in adult, male rabbits. Distraction osteogenesis is used to reconstruct skeletal deformities by fracturing the bone, slightly moving the two segments apart, and allowing new bone to grow in the space. Treatment groups given subcutaneous injections of rHuEpo (4 doses sized 150 IU/kg in 48 hour intervals) had improved bone quality and rate of healing throughout the distraction osteogenesis event (Mihmanli, Dolanmaz et al. 2009). The experimental group also showed greater number of osteoblasts and blood vessels as well as increases in the area of new bone formation (Mihmanli, Dolanmaz et al. 2009). Moreover, reduced osteoclastic activity, increased osteoblastic activity, and significant vascular expansion were observed in comparison to the control group (Mihmanli, Dolanmaz et al. 2009).

Studies have repeatedly shown that Epo improves bone healing, however, the mechanisms regulating the process remain unclear. One proposal is that Epo is able to play a key role in the regeneration of newly resorbed bone by stimulating JAK-STAT signaling pathways in HSCs through Epo-R (Shiozawa, Jung et al. 2010). This then triggers the production of BMPs, most notably BMP2 and BMP6 (Shiozawa, Jung et al. 2010). The resulting production of BMPs by HSCs is able to induce osteogenic progenitor cells to differentiate into osteoblasts and stimulate cartilage formation through the cell surface interactions with BMP receptors (BMPRs) (Shiozawa, Jung et al. 2010). Importantly, Shiozawa et al. demonstrated that Epo serum levels increased during HSC activation while PTH, a hormone known to trigger the formation of bone, did not enhance Epo serum levels (Shiozawa, Jung et al. 2010). The presence of Epo increased the expression of Epo-R as well as caused an increase of BMP2 mRNA in HSCs following *in vitro* culture (Shiozawa, Jung et al. 2010), and in Epo-treated but not control animals (a 4–6-week-old C57BL/6 mouse model) (Shiozawa, Jung et al. 2010). These data suggest that Epo-R expressed by HSCs are functional and can result in the production of BMP2 and BMP6. A second target may be MSCs themselves, for an increase in the expression of Runx2, mineral deposition and enhanced expression of alkaline phosphate activity is observed when bone marrow stromal cells (BMSCs) are treated with Epo (Shiozawa, Jung et al. 2010). Importantly, *in vivo* experiments of both young and mature mice showed increases in the levels of bone mineral density, bone volume fraction, and the number of osteoblasts adhered to bone surfaces when treated with supraphysiologic doses of Epo (Shiozawa, Jung et al. 2010).

Yet the concept that Epo stimulates bone formation is not without controversy. For example, Singbrant *et al.* using a nine-week-old male C57BL/6 mouse model, observed that Epo had

adverse effects on osteogenesis and actually caused a decrease in trabecular bone volume (Singbrant, Russell et al. 2011). They also observed an increase in osteoid volume/bone volume, but they could not detect a change in the rate of mineralization (Singbrant, Russell et al. 2011). *In vitro* cocultures showed that Epo, when administered to osteoblastic cells, had little to no effect on the mineralization potential at varying time points (measured by Alizarin Red staining) (Singbrant, Russell et al. 2011). Furthermore, they did not detect Epo-R expression on HSCs, myeloid cells, or B-lymphoid colonies *in vivo* (Singbrant, Russell et al. 2011). Based on these findings the authors concluded that Epo indirectly caused an increased rate of bone turnover and net loss in trabecular bone. (Singbrant, Russell et al. 2011)

There are several major differences that are noteworthy between the studies that observed bone formation in response to Epo versus those that did not. First is the doses used. Most studies that show bone formation use supraphysiologic doses. Those that did use supraphysiologic doses, often used doses of Epo that fall within a normal range. There may also be differences in the response of animals to Epo based upon age. We ourselves have noted that the response of younger animals to Epo is more robust than is the response in older animals (unpublished observations). Moreover, there are well known differences in the hormonal response of different bones. For example, Singbrant et al. examined the effects of Epo on the proximal tibia (Singbrant, Russell et al. 2011) while our group examined the lumbar vertebrae (Shiozawa, Jung et al. 2010). It is also of interest and important to note that antibody detection of Epo receptors has come under increasing scrutiny of late. In fact, Singbrant *et al.* suggested that anti-body based analysis may not have been as specific as originally thought when it comes to the expression of Epo-R on very pure HSC populations (Singbrant, Russell et al. 2011). However, when purified HSCs were exposed to Epo, JAK-STAT signaling was initiated which lead to down stream production of BMP2 (Shiozawa, Jung et al. 2010). The same strategy was applied to MSC populations, which resulted in biologic activities. The question then arises then how was this observation possible if not through the action of signaling via the Epo-R, unless there are other Epo-Rs which are not recognized by current antibodies. Regardless of the outcome to these controversies, it is clear that the relationship between hematopoiesis and bone formation coupled by Epo is complex and that much work is needed in the area.

Conclusions

There is a growing body of evidence that suggests that hematopoietic molecules and bone formation are linked. Recent and ongoing studies demonstrate that hematopoietic stimulation improves bone formation both in the context of earlier ossification and fraction healing, and improvements in mechanical strength (Ferguson, Alpern et al. 1999; Brager, Patterson et al. 2000; Bozlar, Kalaci et al. 2006; Holstein, Menger et al. 2007). However, the field needs further work before Epo can be used as a therapeutic for bone formation. For example the controversy about the specifics of Epo-R expression and its effects on bone formation still needs to be resolved. Moreover, there is still much to be learned about the molecular mechanisms of Epo and bone formation; are the actions of Epo on the skeleton a coupled event with hematopoiesis, or is it secondary to the actions of Epo on hematopoiesis. If it is a direct action on the skeleton, is the HSC or MSC the target or both, and do both activities need to be present at the same time for bone formation to occur. What is the role of the osteoclast in the process and how are osteoclasts activated. What is the dose and timing needed for Epo to stimulate bone formation and are the doses of Epo safe and effective for use in human trial; if not can other signaling molecules be used in conjunction with Epo for improved therapeutic results. These and other questions will need to be resolved prior to the use of Epo in humans for osseous tissue regeneration.

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Figure 1. Model of Coupled Hematopoiesis and Osteopoiesis

Erythropoietin (Epo) has many roles outside of hematopoiesis. Following hematopoietic stress or acute blood loss, kidney oxygen sensors are activated and to induce Epo secretion. (A) In the bone marrow microenvironment, Epo and BMPs act directly on mesenchymal stem cells (MSCs) and bone marrow stromal cells (BMSCs). This action increases osteoblast formation and activation. Epo's activation of MSCs and BMSCs leads to an increase in bone formation. (B) The increase of Epo in the microenvironment activates and increases cycling of hematopoietic stem cell (HSC) or hematopoietic progenitor cell (HPC). HPC expansion will increase mature blood cell formation and thus recovery of the hematopoietic compartment. (C) This expansion is at the expense of the bone compartment that is reduced by HPC activation of osteoclastogenesis. Moreover, Epo activation of HSC/HPCs induces the secretion of BMPs that further target MSCs to induce osteoblastogenesis for restoration of the bone compartment. The recovery of the blood and bone compartments will act to decrease erythropoietin production by the kidneys.