IBMS BoneKEy. 2008 January;5(1):7-15
http://www.bonekey-ibms.org/cgi/content/full/ibmske;5/1/7
doi: 10.1138/20080293

PERSPECTIVES

Circulating IGF-I and Bone Remodeling: New Insights into Old Questions

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Abstract

The role of insulin-like growth factor-I (IGF-I) in skeletal growth and development has captivated investigators since its discovery fifty years ago. Because IGF-I is ubiquitous yet critical to eukaryotic life, the IGF regulatory system evolved into an exceedingly complex system of ligands, binding proteins, receptors and proteases. Furthermore, both IGFs and the other components of this regulatory system are under the influence of a multitude of genetic, environmental and hormonal determinants. Hence, an answer to one of the most frequently asked question of whether serum IGF-I can predict disease states or determine osteoporotic risk cannot, by its very nature, be simple. *IBMS BoneKEy*. 2008 January;5(1):7-15.
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Introduction

In seminal work that would set the stage for a entire discipline, Salmon and Daughaday reported the presence of a soluble factor induced by growth hormone that had insulin-like properties and mediated somatic growth (1). Subsequent studies over the next half-century would investigate in detail the local and systemic effects of IGF-I. With the advent of an accurate and precise measure of serum IGF-I, circulating levels of this peptide were linked to longevity and to a host of chronic disorders such as cancer, atherosclerosis, osteoporosis, renal failure and Alzheimer's. During the same period, there was a flood of conflicting studies suggesting that circulating IGF-I was purely inert and served only as a storage depot for the import and export of IGF-I. In this review, I will focus on our current understanding of the relationship between IGF-I and the bone remodeling unit, using recent findings from genomic engineering to provide a better appreciation of the role of circulating IGF-I in skeletal physiology and to at least partially address the issue of IGF-I as a risk predictor for osteoporosis.

IGF-I Physiology

The IGFs are 7 kilodalton peptides that regulate cell growth and death (2-6). IGF-I is the predominant adult growth factor, while IGF-II is critical for prenatal development. Bone is the second richest source of IGF-I and IGF-II, and these 'local' IGFs play a major role in skeletal modeling and bone mass acquisition. IGFs are also highly conserved across species; hence the physiology of these peptides can be studied in various animal models. IGF-I and IGF-II differ in their ability to promote tissue growth due to the presence of two distinct IGF receptors (*i.e.*, the type I and type II receptors) (7). IGF-IR is a tetramer consisting of two identical extracellular α-subunits (conferring ligand binding specificity) and two identical transmembrane β-subunits (possessing tyrosine kinase activity). IGF-IR resembles the insulin receptor, and shares amino acid sequence homology (8). IGF-II and insulin also bind to the IGF-I receptor but with 2-15-fold and 1,000-fold lower affinity, respectively (9). IGF1R has intrinsic tyrosine kinase activity critical for specific second message generation, and ligand binding to the extracellular domain of the IGF-IR results in

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autophosphorylation and tyrosine phosphorylation of IGF-IR substrates. Tyrosine-phosphorylated insulin receptor substrate-1 (IRS-1) and SHC bind effector proteins involved in interconnecting pathways, including Ras/Raf-1/mitogen-activated protein kinase (MAPK, also known as extracellular signal-regulated kinase, ERK) (10) and phosphatidylinositol 3-kinase (PI3K)/phosphoinositide-dependent kinase-1 (PDK-1)/Akt. Activation of the Ras/Raf-1/MAPK pathway is critical for cell proliferation whereas the PI3K/PDK-1/Akt pathway is a cell survival signaling system (11). The protein encoded by the Phosphatase and Tensin homolog gene deleted on chromosome Ten (PTEN) is a lipid phosphatase that decreases the activation of Akt by dephosphorylating phosphatidylinositol-3,4,5-triphosphate (PIP3) and phosphatidylinositol-3,4-biphosphate (PIP2). PTEN acts as an "off" switch for the PI3K/PDK-1/Akt pathway as well as a tumor suppressor; mutations of PTEN have been reported in various types of human cancers (12;13). Furthermore, conditional gene deletion of PTEN in osteoblasts results in mice with very high bone mass (14). In contrast to the IGFIR, the monomeric IGF type II receptor (IGF-IIR) binds IGF-II but not insulin, with a 500-fold increased affinity compared to IGF-I. The major physiologic role for IGFIIR is to clear IGF-II from the extracellular space.

There is little ‘free’ IGF-I in the circulation or elsewhere; rather the IGFs are bound to a family of high affinity IGF binding proteins (IGFBPs). There are six IGFBPs (-1,-2,-3,-4,-5, and -6) that belong to the same gene family, although several features (*e.g.*, binding to extracellular matrices) distinguish one from the other (15). The binding affinity of IGFBPs to IGFs is greater than that of the IGF receptors themselves, hence the IGFBPs can block the interaction between IGF-I and the IGFIR (16;17). Nevertheless, binding of IGFBPs to IGFs can also protect the IGFs from proteolytic degradation, and consequently enhance IGF actions by augmenting their bioavailability in local tissues (16;17). The IGFBPs also may serve to deliver IGF-I to the IGF1R. Therefore, the IGFBPs can act as either agonists or antagonists for IGF-I, and their ultimate impact on the IGFs also depends to a large extent on their post-translational modification by phosphorylation and the degree of proteolysis (16-19).

The IGFBPs also have IGF-independent properties, some of which may occur at the remodeling unit (16;20). IGFBP-3 was the first binding protein demonstrated to have effects on cell growth, independent of IGF-I. IGFBP-2 was recently shown to suppress PTEN activity in tumor cells by binding to the integrin receptor (21). Our investigative group, in collaboration with David Clemmons at UNC-Chapel Hill, demonstrated that IGFBP-2, independent of IGF-I, can suppress PTEN activity in osteoblasts and osteoclasts (22). If supported by future *in vivo* studies, these data would suggest there may be another mechanism whereby the IGF regulatory system affects differentiation and/or programmed cell death. In a similar vein, PAPP-A, a serine protease that cleaves IGFBP-4 and other peptides, when genetically deleted, produces a skeletal phenotype of low bone mass, presumably due to decreased IGF-I availability (Conover, personal communication). Hence, the IGF regulatory system can be affected in numerous ways and cause significant changes in the growing skeleton.

What role, if any, does circulating IGF-I play in skeletal growth and maturation? To answer that question, the physiology of the IGF ternary complex needs to be fully understood. First, the distribution of IGFs in the circulating pool is determined by the relative saturation of the IGFBPs, and the composition of a ternary complex. This 150 kD complex is composed of IGF-I (and -II), IGFBP-3 (or IGFBP-5), and the acid labile subunit, ALS, a 70kD protein. Normally, the vast majority of circulating IGF is bound to this saturated intravascular complex, whereas the IGFBPs are unsaturated and have significant binding capacity. Translocation of IGF-I out of the circulation can only occur by the transcapillary transport of unbound IGF-I, or by movement of IGF-I bound to the low molecular weight

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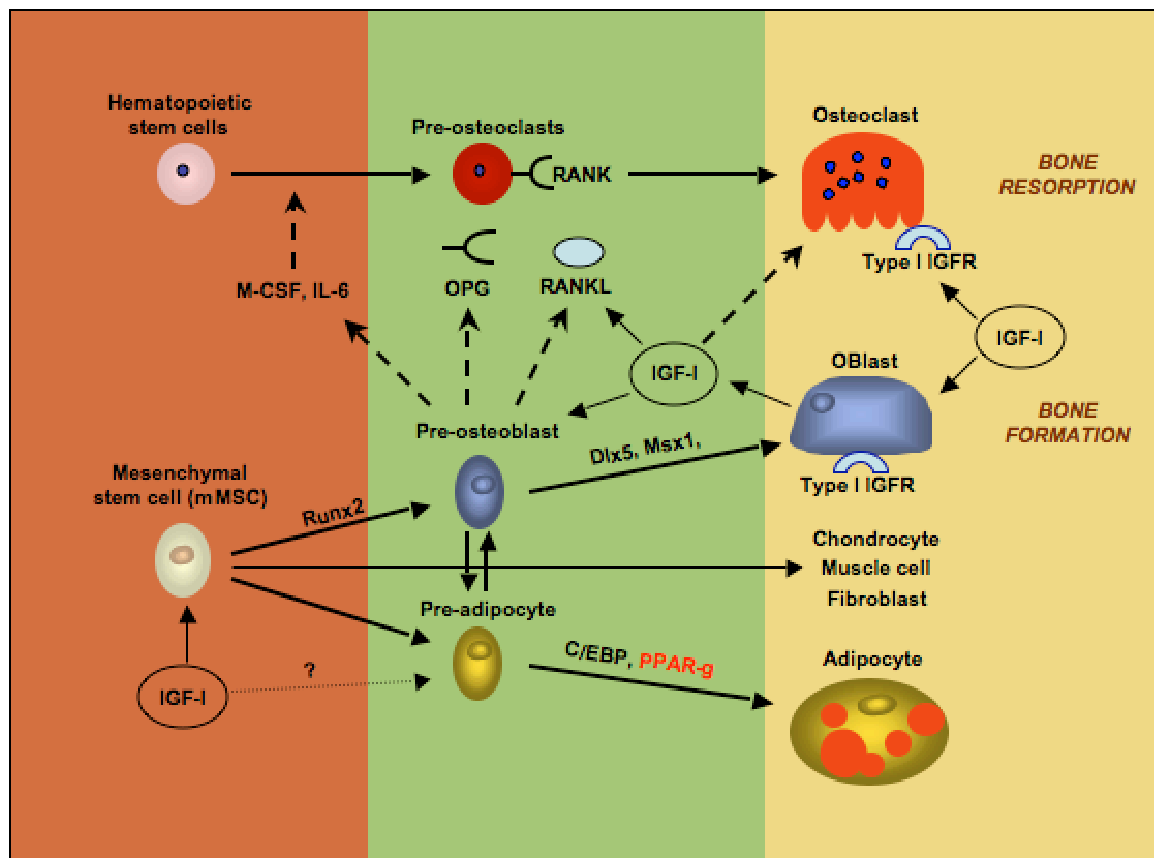

Figure 1. Bone remodeling requires recruitment of osteoblasts and osteoclasts in a finely orchestrated manner. IGF-I is important in the differentiative function of the osteoblast (Oblast) as well as being synthesized by these cells. IGF-I is also important in osteoclastogenesis and may be a critical factor in early adipogenesis. The stem cell stage is depicted in the left column, the pre-osteoclast, -osteoblast, and -adipocyte stage in the middle column, and the differentiated cell in the right column.

IGFBPs from blood to the interstitium. Infusions of IGF-I can produce a transient rise in free IGF-I, and therefore some delivery to tissue sites. But co-incident with these changes, there is suppression of IGF-II, insulin, and endogenous growth hormone (GH). As such, during the course of IGF-I treatment, IGF-I is partitioned into these pools so that some IGF-I saturates the ternary complex and stays in the circulation. The remaining IGF-I goes into the lower molecular weight (50 kDa) unsaturated IGFBP pool where transport into the extravascular space is possible. Partitioning of IGFs into the various binding fractions is protective yet critical for the biologic activity of IGF-I. It may also explain why treatment with IGF-I may not increase circulating levels in rodents and may have distinct tissue effects compared to therapy with growth hormone that results in a predictable rise in IGF-I, ALS and IGFBP-3. Since it has been demonstrated that IGF-I infusions can stimulate bone turnover, it is clear that components of the circulating IGF-I complex are not inert and are important physiologic regulators.

IGF-I and the Bone Remodeling Unit

Bone formation and remodeling require the integration of multiple signaling pathways, which in turn regulate the activities of lineage-specific master genes as well as their cellular substrates (11). Despite the importance of the IGFs in cell growth and homeostasis (*i.e.*, cell cycling and apoptosis), their precise role in osteoblast

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and osteoclast differentiation is still a matter of debate. In part this relates to the complex, yet developmentally sensitive and finely orchestrated process that drives mesenchymal stem cells into the bone lineage (see Figure 1). Not unexpectedly, this pathway involves multiple transcription factors and cytokines, as well as the IGFs. The most notable transcription factors that control osteoblast fate are Runx2 and Osterix (Osx). Runx2 (runt-related transcription factor 2)/Cbfa1 (core binding factor 1)/Pebp2A (polyoma enhancer binding protein 2A), a transcription factor that belongs to the Runx family, is the α subunit of a heterodimeric transcription factor, PEBP2/CBF, which is composed of α and β subunits (23;24). *Runx2* is required for osteoblast differentiation and bone formation; gene deletion results in embryonic lethality with the notable absence of a skeleton. Runx2 also functions later in osteoblast differentiation to regulate the formation of the extracellular matrix (25). IGF-I can regulate Runx2 expression by activation of the PI3K, Pak1, and ERK pathways (26;27).

Osx is a master zinc-finger-containing transcription factor of osteoblast lineage progression that is highly specific to osteoblasts *in vivo* (28). It acts downstream of BMP-2/Smad signaling (26;27). The Osx amino acid sequence predicts three C2H2-type zinc fingers that have a high degree of identity to similar DNA-binding domains in the transcription factors Sp1, Sp3 and Sp4. The expression of Osx is more specific to osteoblasts than Runx2 (29). Because no Osx transcripts are detected in skeletal elements of Runx2-null mice, Osx must be downstream of Runx2 in the pathway of osteoblast differentiation (28). Moreover, both IGF-I and BMP-2 are shown to up-regulate Osx expression during early osteoblast differentiation (30). In mesenchymal stem cells, it appears that both MAPK and PKD signaling pathways serve as points of convergence for mediating the IGF-I- and BMP-2-induced effects on Osx expression (31). IGF-I-mediated Osx expression requires all three MAPK components (Erk, p38, and JNK), whereas BMP-2 requires p38 and JNK signaling, and the synergistic interactions of BMP-2 and IGF-I are also disrupted by PKD inhibition (31).

It should be noted that Runx2-independent pathways of ossification may exist, including (1) the Wnt signaling pathway; (2) the Msx2-dependent vascular ossification pathway; and (3) Osx induction via Dlx5, a homeobox transcription factor, which acts downstream of BMP-2 (30;31). These studies suggest additional pathways may act in parallel to, or independent of Runx2 to regulate Osx expression during osteogenic lineage progression. Indeed, in the canonical Wnt signaling pathway, there is likely to be cross-talk with the IGF pathway. For example, β-catenin binds to IRS-1 (as well as other factors), to enhance its transport from the cytoplasm into the nucleus, where it can affect a cascade of downstream target genes.

In sum, IGFs enhance late differentiation of osteoblasts. In contrast, their mitogenic role in the skeleton is relatively limited. However, IGF-I may also be important in lineage allocation, or in promoting adipogenesis within the marrow niche independent of other known transcription factors. Mesenchymal stem cells can enter several distinct lineages, and the precise timing of IGF-I action in this process requires further investigation.

IGF-I affects the remodeling unit not only through modulation of the osteoblast, but also the osteoclast. Recent studies have been particularly insightful. The IGF1R is expressed on osteoclasts and on some hematopoietic precursor cells. Recombinant IGF-I stimulates osteoclastogenesis *in vitro*, and RANKL, the cytokine necessary for osteoclast differentiation, is enhanced in marrow stromal cells. *In vivo* administration of IGF-I, or growth hormone, to rodents or humans leads to increased bone turnover including both formation and resorption. This would make IGF-I a remodeling stimulus (30).

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Genetic Manipulation of the IGF Regulatory System and the Skeletal Consequences

Genomic engineering has the capability to define the role of skeletal and circulatory growth factors in respect to bone acquisition and remodeling. Indeed, each of the components of the IGF regulatory system has been deleted or over-expressed either globally or conditionally in bone. A comprehensive review of all of these studies is beyond the scope of this review. Suffice it to say that findings from these studies have been surprising, albeit illuminating. For example, global deletion of the *Igf1* gene generally results in postnatal death due to skeletal muscle insufficiency. However, about 20% of the pups survive, due in part to tremendous increases in GH secretion, as a compensation for the absence of IGF-I. The adult *Igf1* gene-deficient mouse is generally very small, with short femurs, reduced cortical bone, but greater trabecular elements in the vertebrae and distal femur (32). Marrow stromal cells from these mice do not proliferate or differentiate *in vitro* to the same degree as wild type controls. However, the most compelling feature of this mutant is the trabecular bone phenotype, which has been somewhat of a paradox. Interestingly, deletion of the *Als* gene in mice results in a similar but more dramatic phenotype whereby the cortical elements are markedly diminished but the trabecular component is increased. Both mutants (*Igf1*(-/-) and *Als*(-/-)) are unresponsive to PTH (33). Preliminary data from the Yakar and Rosen laboratories also suggest that in both mutants, the magnitude of osteoclast differentiation in response to mCSF and RANKL is less than controls. These data imply that despite secondary increases in growth hormone, the *Igf*1 mutants have an intrinsic defect in osteoclastogenesis. Whether this is cell autonomous or not remains to be determined. Similarly, the decline in circulating IGF-I with PTH treatment in rodents (Jilka R, personal communication) will require further studies, although it may imply greater tissue delivery, rather than increases in the ternary complex. However, in humans treated with PTH for osteoporosis, IGF-I levels are unchanged.

Global deletion or transgenic expression of the IGFBPs has been successfully accomplished, but the results are less clear than expected. For example, IGFBP-2 transgenics tend to have shorter femurs and stunted growth, while the global *Igfbp2* knockout is characterized by reduced bone turnover and impaired bone formation. IGFBP-5 transgenics have low bone mass even though this binding protein is considered an agonist for IGF-I. On the other hand, gene deletion of *Igfbp3* results in greater bone turnover, albeit lower bone mass. Faced with drawing conclusions from genetically engineered mice, two factors become evident. First, there is a tremendous degree of compensation that occurs with deletion or overexpression of IGF components in mice. These changes can overshadow a primary skeletal phenotype that results from the initial germ line event. Second, deletion or over-expression of a single IGFBP can produce a phenotype that may not reflect the true function of that peptide, in part because the relative proportion of IGF-I to the respective IGFBP is a critical part of the action of the latter. So, for example, with IGFBP-2, over-expression of this protein results in low bone mass, but if there is co-incident administration of IGF-I, high bone mass would likely occur.

Another genetic approach for understanding the function of a particular growth factor, binding protein, or cytokine, is to delete or over-express an IGF regulatory component in the skeleton only. For example, Zhang *et al*. used a Cre/loxP system to delete the IGF-IR in mature osteoblasts (34). These investigators found that mice with the receptor knockout had a significant impairment in mineralization lag time but adequate numbers of osteoblasts. Similarly, targeted over-expression of IGF-I in osteoblasts resulted in mice with increased differentiative function (*e.g.*, greater collagen synthesis), more osteoclasts with greater bone resorption, but no change in the number of osteoblast precursors. *In vitro* studies have demonstrated that IGF-I can increase type I collagen synthesis, alkaline phosphatase activity and osteocalcin production in osteoblasts (35;36). IGF-I also

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acts as a potent anti-apoptotic factor particularly for osteoblasts and osteocytes, probably via the PI3's kinase signaling pathway. On the other hand, Rowe *et al*. used timed microarray technology with a GFP Col3.6 promoter and showed that during marrow stromal cell recruitment, IGF-I expression declined, particularly as these cells entered the osteoblast lineage (37). Taken together, these data suggest that IGF-I is very important in the late stages of osteoblast differentiation, and less important for the recruitment of osteoblast precursors.

Deletion or overexpression occurs as an engineered germ line alteration. Hence the defect is established from the time of embryogenesis and is present throughout life. This makes it very difficult to determine when skeletal growth factors exert their most important effects on the skeleton. Introduction of a conditional allele for the *Cre-recombinase* gene that can be activated by specific compounds such as tamoxifen, RU-486 or tetracycline, with a bone- or liver-specific promoter, can begin to address these and other questions. For example, the Yakar laboratory recently demonstrated that a liver-specific inducible deletion of the *Igf1* gene at 4 weeks of age using a single dose of tamoxifen resulted in a significant skeletal phenotype with reduced cortical bone mass at 8 and 16 weeks of age. On the other hand, gene deletion at 8 or 16 weeks of age had much less of an impact on the cortical skeleton. Not surprisingly, these findings would suggest that the genes regulating peak bone acquisition are turning on very early in life, around the time of weaning, or sooner. More importantly, in our quest for identifying allelic determinants of these genes, it is clear that developmental timing for the onset or offset of particular genes is of critical importance.

Genetic engineering in mice and humans can also occur spontaneously in nature as mutations. For example, our laboratory found a very small mutant mouse that was born from a normal mother in a regular colony. *Small (Sm)*, as it was subsequently named, arose from a homozygous recessive mutation. The defect was mapped to mouse chromosome 1 and subsequently identified as being a nonsense mutation in the *Irs1* gene, leading to a truncated protein that could not signal properly. Besides its small size, *sm/sm* also had a pronounced skeletal phenotype including reduced bone mass (both cortical and trabecular), and markedly impaired bone formation, but surprisingly, normal numbers of osteoblasts and osteoclasts. Interestingly, the genetically engineered *Irs1*(-/-) mice also are short, do not respond to PTH and have low bone mass (38). But unlike *sm/sm*, these mice had reduced numbers of both osteoblasts and osteoclasts. Both these strains will be useful in understanding the downstream consequences of impaired signaling from the IGF1R. But, they also highlight two caveats. First, compensatory changes that permit survival may also affect skeletal integrity. Second, the genetic background is important for understanding the skeletal phenotype. In this case, the two mutants differed in their genetic background (*i.e.*, strain). Thus, gene by gene interactions are a critical aspect of mouse and human physiology and these effects will require more studies.

Summary

IGF-I is an important growth factor for the skeleton, primarily because it affects the differentiative function of osteoblasts. But, the other components of the IGF regulatory system can also impact bone development in both an IGF-I-dependent and -independent manner. Circulating IGF-I is important for cortical integrity, probably by stimulating periosteal expansion. There may be a role for circulating IGF-I in modulating trabecular acquisition but this is not clear. The effects of IGF-I on osteoclastogenesis require further study. In terms of fracture risk prediction, although we have a solid working knowledge of IGF-I, the dynamic of the IGF-I circulating complex is such that conclusions based on correlative data should be considered only in the context of the many variables associated with *in vivo* analyses.

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Acknowledgment: This work was supported by PHS NIAMS; AR53853

Peer Review: This article has been reviewed by Serge Ferrari and René Rizzoli.

Conflict of Interest: None reported.

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