Published in final edited form as:
Growth Horm IGF Res. 2007 June ; 17(3): 179–185.

Insulin-like growth factor binding protein-5 in osteogenesis: Facilitator or Inhibitor?

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Abstract

The insulin-like growth factors (IGFs) play a central role in controlling somatic growth in mammals and exert anabolic effects on most tissues, including bone. IGF action is mediated by the IGF-I receptor and additionally is regulated by six high-affinity IGF binding proteins (IGFBP-1 through IGFBP-6), of which IGFBP-4 and IGFBP-5 are most abundant in bone. The focus of this brief review is on the role of IGFBP-5 in bone biology. IGFBP-5 has been implicated as a pro-osteogenic factor in several studies but conversely has been shown to act as an inhibitor of bone formation, primarily by interfering with IGF actions on osteoblasts. These potentially contradictory effects of IGFBP-5 in bone are further complicated by observations indicating that IGFBP-5 additionally may function in an IGF-independent way, and may have been accentuated by differences in both experimental design and methodology among published studies. Suggestions are made for a more systematic approach to help discern the true roles of IGFBP-5 in bone physiology.

Introduction

Skeletal development and remodeling are controlled by systemically-derived and local signals mediated by protein hormones, peptide growth factors, and other biologically-active molecules. Among growth factors with actions on bone are the insulin-like growth factors, IGF-I and -II. These molecules are closely-related single-chain secreted proteins that bind with high affinity to the IGF-I receptor (IGF-IR) on the surface of responsive cells, and to a family of soluble IGF binding proteins, IGFBP-1 through -6. It is generally agreed that the IGF-IR, a membrane-spanning ligand-activated tyrosine protein kinase, mediates the biological actions of the two IGFs, while the IGFBPs play more modulating roles, potentially by regulating both IGF half-life and access to the IGF-IR [1–3]. Recent studies additionally support the idea that IGFBPs also may have biological actions that are independent of their ability to bind IGF-I or IGF-II [4], although aspects of this research remain controversial. Among the six IGFBPs, IGFBP-4 and -5 are the most abundant in bone [4,5].

In this review we will focus on the actions of IGFBP-5 in bone. Results from a variety of studies have been interpreted to support the hypothesis that IGFBP-5 enhances bone formation and osteoblast differentiation, while alternatively other experiments have suggested that IGFBP-5 inhibits osteoblast functions, primarily by blocking the effects of IGF-I and IGF-II on these cells. We will discuss each side of this controversy, and will highlight differences in both

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experimental design and methodology that might explain the conflicting results. Finally, we will offer suggestions for a more systematic approach to help elucidate the true actions of IGFBP-5 in bone physiology.

IGFBP-5: Structural Considerations

IGFBP-5 is a 252 amino acid secreted protein that consists of highly conserved, cysteine-rich, NH_2- and COOH-terminal domains, and a less well-conserved central segment [1,6,7]. It has been found that the NH_2-terminal domain of IGFBP-5 contains the primary IGF binding site, and mutagenesis experiments directed toward this region of the molecule have demonstrated that 27 amino acids, from valine 49 to leucine 75 of the mature protein, form a hydrophobic patch on the surface of IGFBP-5 that is necessary for IGF binding [1,8]. As shown by NMR spectroscopy, the isolated NH_2-terminal segment of IGFBP-5 forms a tight globular structure with three strands of anti-parallel β sheets in the center [9]. The structure of the full-length protein has not been characterized yet.

The COOH-terminal domain of IGFBP-5 contains 6 conserved cysteine residues and binds via a series of basic amino acids to extracellular matrix components such as heparin and hydroxyapatite [10–12]. This segment of the molecule also contains a binding site for acid-labile subunit (ALS), the protein that forms a ternary complex with either IGFBP-3 or IGFBP-5 and IGF-I in the blood [13,14]. A region of basic amino acids within the COOH-terminal region also has been shown to act as a putative nuclear localization sequence in over-expression experiments, but it remains unknown if nuclear accumulation of IGFBP-5 occurs under normal physiological conditions [1,6]. The isolated COOH-terminus of IGFBP-5 cannot bind IGF-I or IGF-II by itself but mutagenesis studies indicate that it contributes to the overall affinity and stability of ligand binding in the full-length protein [15,16]. As the three-dimensional structure of full-length IGFBP-5 has not been resolved yet, the physical nature of interactions between the NH_2- and COOH-terminal segments of the protein to define the high-affinity IGF binding site remains conjectural.

The linker (L) domain of IGFBP-5 separates the NH_2- and COOH-terminal segments. The L domain contains several potential proteolytic cleavage sites, and is a substrate for at least two distinct proteases that reside in the extra-cellular environment of osteoblasts, matrix metalloprotease-2 and ADAM-9 [17,18]. IGFBP-5 also undergoes several post-translational modifications. Within the L domain are three predicted O-linked glycosylation sites [19], and 12 putative phosphorylation sites [20], although the full extent of phosphorylation has not been established in any tissue in which IGFBP-5 is produced and secreted.

IGFBP-5 and Bone

IGF action enhances bone mass

A number of studies in experimental animals have concluded that IGF action is essential for normal bone formation, growth, and maintenance. For example, mice lacking the IGF-IR in all cell types have retarded skeletal development accompanied by delayed ossification, as well as many other severe systemic defects that contribute to their neonatal death [21]. Targeted loss of the IGF-IR exclusively in osteoblasts also has a bone phenotype. In these mice, bones were of normal length but total trabecular volume was reduced by nearly 50%. The main defect appeared to be a decline in mineral apposition rate [22]. In agreement with the conclusions of these studies, targeted over-expression of IGF-I in mature osteoblasts in mice via the osteocalcin promoter caused an increased rate of bone formation and more extensive mineralization than normal, which resulted in a ~30% increase in trabecular bone volume compared with controls [23]. Targeting IGF-I to osteoblast precursor cells in mice with the rat collagen type I gene promoter also gave rise to a robust bone phenotype, and led to increases

in femur length, cortical width, and cross-sectional area [24]. Thus, regardless of the timing of IGF-I over-expression in bone in transgenic mice, net bone formation is enhanced. Therefore, based on several types of evidence, IGF action via the IGF-IR is critical for normal bone development and mineralization.

Many of the effects of growth hormone (GH) on bone are thought to occur as the result of its ability to stimulate the synthesis of IGF-I throughout the body, including in osteoblasts [25]. In agreement with this concept, mice lacking the GH receptor in all tissues have diminished cortical bone growth and trabecular turnover at 6 weeks of age, which could be reversed within two weeks by systemic IGF-I treatment [26].

Expression of IGFBP-5 in bone cells

Soon after its initial discovery, IGFBP-5 was isolated from human bone [27]. During fetal development, it is expressed during endochondral bone formation in differentiating chondrocytes and osteoblasts [28,29], while in primary osteoblast cultures, IGFBP-5 is secreted by pre-osteoblasts but decreases in abundance during their differentiation and maturation [30]. The accumulation of IGFBP-5 also varies in cultured osteoblasts derived from different bone sources, being more abundant in conditioned media from calvarial osteoblasts than in media from cells isolated from the mandible, vertebral bodies, or ribs [31]. It has been proposed that site-dependent differences in IGFBP-5 production by different bones are secondary to alterations in either the growth factor micro-enviornment or in the rate or extent of mechanical loading, but it is additionally possible that osteoblasts from different locations respond differently to the same cues in culture. Many bone cell lines also express IGFBP-5 at varying levels [32].

The production and accumulation of IGFBP-5 in bone cells appears to be regulated by multiple factors [33]. Several hormones, including prostaglandin E_2 and parathyroid hormone, enhanced IGFBP-5 gene expression in cultured osteoblasts [34-36], while transforming growth factor-β, platelet-derived growth factor, and basic fibroblast growth factor each reduced IGFBP-5 mRNA abundance in the same cells [37]. IGF-I also has been shown to induce IGFBP-5 mRNA and protein synthesis in cultured osteoblasts [19,33,34,37] and in bone in transgenic mice over-expressing IGF-I in osteoblasts [38]. The amount of IGFBP-5 in bone matrix also may be regulated by its proteolysis [17], which appears to be blocked upon binding of IGF-I.

Effects of IGFBP-5 on bone formation

Positive outcomes—Systemic administration by daily subcutaneous injection of equimolar amounts of IGFBP-5 and IGF-I in combination for 8 weeks led to a 20% increase in cortical bone formation, bone area, and mineral density in mice [39]. Similarly, a 40% rise in local bone area and density was seen after a 19 day course of daily injections of both IGFBP-5 and IGF-I into calvariae (67 or 200 μg/day of IGFBP-5). By contrast, the same doses of IGFBP-5 alone had no effect [40]. However, in other studies, daily subcutaneous injections of *E. coli* derived IGFBP-5 (50 μg/day for 20 days) in mice led to increases in serum osteocalcin levels (58%) and in femoral bone alkaline phosphatase activity (85%), although direct effects on bone formation were not assessed [41]. It is important to note that systemic treatment with IGF-I or IGFBP-5 may lead to effects on bone that are caused by other factors that may be regulated by either protein. In addition, as IGFBP-5 may undergo proteolytic cleavage after systemic administration, it may not have the same effects on bone as local delivery of the protein. In this regard, observations with IGFBP-4 are instructive, as the protein was rapidly degraded after systemic injection, leading to increased bone formation, while when delivered locally to bone or added to cultured osteoblasts, it was inhibitory [42].

In one group of *in vitro* experiments, *E. coli*-derived IGFBP-5 (100 ng/ml) was shown to increase DNA synthesis in the MC3T3 pre-osteoblastic cell line and in primary mouse osteoblasts up to 4-fold compared with vehicle-treated controls, and was equally effective with or without added IGF-I [43]. Similar results were seen using human cell culture-derived IGFBP-5, although a much higher dose was needed (2300 ng/ml), suggesting variability in potency or stability between bacterial and mammalian cell derived IGFBP-5 [16]. Although cell counts were not performed in these experiments, these observations have been interpreted to indicate that IGFBP-5 was able promote osteoblast proliferation.

Very few experiments have shown positive effects of IGFBP-5 on osteoblast differentiation in culture. Treatment of the MG63 osteoblastic cell line or primary human osteoblasts with *E. coli*-derived IGFBP-5 resulted in modest increases in alkaline phosphatase activity and osteocalcin protein levels [41]. Similar results were observed in mouse osteosarcoma cells transfected with an expression plasmid for IGFBP-5 [44], but no longer-term outcomes were assessed, such as effects on mineralization.

Negative outcomes—Osteoblast-specific over-expression of IGFBP-5 in transgenic mice via the osteocalcin promoter resulted in small decreases in bone mineral apposition rate and in trabecular bone volume by 4–5 weeks of age that were normalized by 24 weeks, coincident with the drop in osteocalcin promoter activity with aging [45]. Even though mineralization was reduced, osteoid width was unchanged, which resembled the phenotype seen in mice lacking the IGF-IR in osteoblasts [22]. As the number of osteoblasts was normal in these mice and osteoclast number and activity were identical to controls [45], the results indicate that IGFBP-5 impaired osteoblast function. Cultured bone marrow stromal cells from these mice also showed diminished mineralization.

In another transgenic mouse model, IGFBP-5 was expressed in multiple tissues via the β-actin gene promoter, leading to up to an 8-fold increase in serum levels compared with controls [46]. Long bone mineral density, as assessed by dual energy X-ray absorptiometry and quantitative computed tomography, was reduced by 30% by 3 weeks of age, although these effects were observed primarily in male mice, where accumulation of IGFBP-5 in the blood was highest. Based on these results, it appears that over-expressed IGFBP-5 reduces net bone mineralization. However, as neither osteoblasts nor osteoclasts were examined directly in these experiments, it is unclear whether the effects on bone were caused by changes in their numbers, function, or both.

Other results using cell culture models support the idea that IGFBP-5 inhibits bone formation. Over-expression of IGFBP-5 in osteosarcoma cells reduced their proliferation by 30% [44]. In another study, addition of mammalian cell culture-derived IGFBP-5 to U2 osteosarcoma cells blocked proliferation induced by IGF-I but not by a derivative, des-1,3-IGF-I, which interacts poorly with IGFBPs [19]. In addition, over-expression of IGFBP-5 in MC3T3 cells using a retroviral vector caused a decline in differentiation as measured by reductions in osteoblast-specific mRNAs and proteins, and decreased mineralization [47].

Bone density is dynamically controlled through contrasting actions of osteoclasts and osteoblasts in the bone microenvironment. Yet only a few studies have examined the effects of IGFBP-5 on osteoclasts. In one group of experiments, IGFBP-5 caused an increase in osteoclast formation and stimulated a 7-fold rise in bone resorbing activity [48], lending further support to an overall inhibitory role for IGFBP-5 in bone.

Evidence for IGF-independent effects of IGFBP-5 in bone

As outlined in preceding sections, IGFBP-5 can play a modulating role on the primarily anabolic effects of IGF-I or IGF-II in bone. There is also some evidence for IGF-independent

actions of IGFBP-5 on bone cell function. Major support for this hypothesis derives from studies in osteoblasts isolated from IGF-I-deficient mice. In one series of experiments, addition of *E. coli*-derived IGFBP-5 resulted in small increases in cell proliferation (< 40%) and in early indicators of differentiation, such as alkaline phosphatase activity (< 40% rise) [40]. Since IGF-II was undetectable in the conditioned media from these cells, the results were interpreted as IGF-independent effects of IGFBP-5. Small increases in alkaline phosphatase activity also were measured in parietal bones of IGF-I knockout mice after IGFBP-5 was injected into the periosteum [41].

Several potential mechanisms have been postulated to be responsible for these putative IGF-independent effects of IGFBP-5 in bone. IGFBP-5 has been shown to bind to an uncharacterized cell surface protein of ~ 450 kDa in osteoblasts [49], but it remains to be determined whether this is a receptor, as to date no IGFBP-5-stimulated intracellular signaling pathways have been identified. It also has been suggested that IGFBP-5 encodes a potential nuclear localization sequence within its COOH-terminus [6], and in one series of studies, after exogenous administration, IGFBP-5 could be detected in the nucleus of human osteoblasts by immunocytochemistry [17]. Whether nuclear accumulation of IGFBP-5 occurs *in vivo* under physiological conditions remains unknown, and the biological consequences remain to be characterized. Genetic screens using the yeast 2-hybrid assay with IGFBP-5 as ‘bait’, and a cDNA expression library from U2 osteosarcoma cells as ‘prey’, have led to the identification of several putative IGFBP-5 interacting proteins that are primarily expressed intracellularly [4]. At present, despite some intriguing initial observations, the physiological relevance of these interactions and their roles in mediating the effects of IGFBP-5 bone remain uncharacterized.

Summary and perspectives for the future

In this review we describe the potentially conflicting roles for IGFBP-5 as both a facilitator and an inhibitor of bone formation and osteoblast function. In one group of observations, it was shown that IGFBP-5 could collaborate with IGF-I to increase bone formation and enhance bone mineral density in mice [39]. The evidence that IGFBP-5 was effective on its own *in vivo* was less compelling, as parameters of bone formation were not measured directly in the studies reporting positive outcomes [40]. Conversely, in other experiments, over-expression of IGFBP-5 targeted to osteoblast precursor cells, or delivered systemically, led to a decline in bone formation in mice, and in one group of studies, caused a reduction in long bone mineral density [45,46]. Results of experiments in cultured cells were equivalently contradictory, in some cases showing that IGFBP-5 could increase osteoblast DNA synthesis [16,40,43], and in others demonstrating a decline in cell proliferation [19,44]. Other results showed either increases or decreases in parameters of osteoblast differentiation [40,41,44,47].

How can we explain these very divergent and confounding observations? One possibility is differences in experimental design. As seen in Table 1, this includes use of different osteoblastic cell lines, which may vary in terms of IGF-I receptor expression, in the production and secretion of IGF-I, IGF-II, other growth factors with effects on bone, or other IGFBPs, to name just a few differences. Also, with regard to *in vivo* experiments, systemic versus local delivery of IGFBP-5 in mice almost certainly will cause variability in outcomes, as will continuous IGFBP-5 over-expression as seen in transgenic models when compared with the intermittent delivery that occurs after repeated injections. Additionally, the source and the extent of purification of exogenous IGFBP-5 could cause variability. IGFBP-5 derived from an *E. coli* production system may behave differently than IGFBP-5 purified from mammalian sources, in part because of differences in post-translational modifications. In this regard, a comparative study of dose-dependent biological effects of all sources of recombinant IGFBP-5 would be very instructive.

Other reasons for contradictory outcomes may relate to the parameters being measured. While it should be obvious that increases in rates of DNA synthesis do not necessarily equal alterations in cell number, often the distinctions are blurred. Similarly, changes in serum levels of proteins made in bone are not equivalent to alterations in the extent of new bone formation or changes in bone mineral content measured directly. Suggestions for a more systematic and uniform approach to evaluating the actions of IGFBP-5 in bone are listed in Table 2.

Finally, while it is clear that IGFBP-5 and other IGFBPs influence the biological effects of IGF-I and IGF-II, it is still unproven how IGFBP-5 might act through IGF-independent mechanisms in bone or in other tissues. Experiments to prove IGF-independent effects of IGFBP-5 are very difficult to perform convincingly, and results to date remain subject to alternative interpretations. If IGFBP-5 acts through a cell-surface receptor, then the responsible signaling pathways need to be characterized. Similarly, if IGFBP-5 acts in the nucleus, then it needs to be shown in as compelling a way as possible that it enters the nucleus under physiologically-relevant conditions, and does not accumulate there secondary to over-expression of either native or modified versions of the protein in cultured cells.

IGFBP-5 is the most conserved of the six IGFBPs, and appears to be present in all vertebrate species which have been examined. It is time to define its actions in bone and in other tissues as completely as possible.

Acknowledgements

This review was supported in part by US National Institutes of Health research grants, RO1 DK63073 and RO1 DK42748 (to P. R.).

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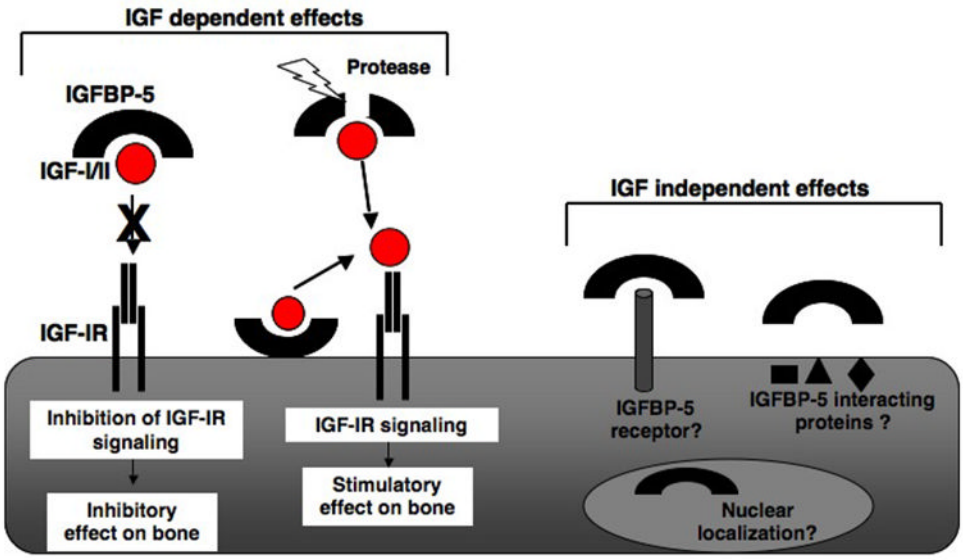

Figure 1.
Effects of IGFBP-5 in bone. Illustrated are potential mechanisms by which IGFBP-5 modifies the actions of IGF-I and IGF-II in bone cells. Also shown are putative IGF-independent effects of IGFBP-5. See text for details.

Table 1

Effects of IGFBP-5 on bone parameters

	System	Source of IGFBP-5	Effects observed
Stimulatory effects	Mouse osteoblasts in primary culture	Mammalian cell culture	Increase in thymidine incorporation in
	MC3T3, human osteoblasts in primary culture	*E. coli*-derived	Increase in thymidine incorporation in
	MG63, human osteoblasts in primary culture	*E. coli*-derived	Increases in osteocalcin and alkaline p
	Wild type and IGF-I-deficient mice	*E. coli*-derived	*In vitro* - increase in thymidine incorp
	In vitro-calvarial osteoblasts		*In vivo* - increase in alkaline phophata
	In vivo-injection in parietal bones		
	Local or systemic injections of IGFBP-5 and IGF-I in rats	*E. coli*-derived	Increase in cortical bone formation, bo
	Mouse osteosarcoma cells (OS/50K8)	Expression plasmid	Increase in osteocalcin mRNA
Inhibitory effects	U2 osteosarcoma cells	Mammalian-cell culture	IGF-I-mediated cell proliferation inhib
	Mouse osteosarcoma cells	Expression plasmid	Increase in cell doubling time (decreas
	MC3T3	Retrovirus	Decreased mRNAs of osteoblast speci
	IGFBP-5 transgenic mice	Systemic expression via β-actin promoter	Decrease in bone mineral density and
	IGFBP-5 transgenic mice	Osteoblast-specific expression	Transient decrease mineral apposition;

Table 2

Guidelines for evaluating IGFBP-5 actions in bone

Parameter	Guidelines
IGFBP-5	Source (bacterial vs. mammalian derived) • Stability • Biological potency (IGF binding, other assays) Mode of delivery • Exogenous addition vs. over-expression (cell culture) • Systemic vs. local injection vs. osteoblast-specific targeting (*in vivo*)
Cell culture system	Stage of differentiation (pre-osteoblasts vs. osteoblasts) Abundance of IGF system components (IGF-IR, IGF-I and -II, IGFBP-5 proteases, other IGFBPs) Expression of other factors capable of regulating IGFBP-5
Assessment of osteogenic outcomes	*In vitro* Proliferation - DNA synthesis rates, cell counts, cell doubling time Differentiation - time course studies with standardization of osteogenic markers (gene expression, protein levels, enzymatic assays, etc.) Mineralization rates and extent *In vivo* Analysis of local bone parameters (bone mineral density, cortical and trabecular bone, mineral apposition rates, etc.) Effects on different bone cell types (osteoblast, osteoclast, osteocyte)