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Bone-Derived Factors Active on Bone Cells

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Summary. Effects of systemic calcium regulating hormones have been studied extensively, yet mechanisms of bone volume regulation at the local level are poorly understood. Our laboratory has reported evidence for two locally mediated processes of bone volume regulation which function independently of systemic control: (1) coupling of bone formation and resorption and (2) repletion of resorbed bone. These local regulatory mechanisms have been shown to occur *in vivo* and *in vitro*. We have reported that embryonic chick tibiae in culture, stimulated to resorb, release a factor in the serum-free culture medium that stimulates bone cell proliferation and bone matrix formation *in vitro*. We have postulated that this factor could be involved in the coupling mechanism. Subsequently, a similar factor which stimulates bone cell proliferation, collagen synthesis and bone formation *in vitro* was extracted from embryonic and adult bones. The factor partially purified from human bone, designated as human skeletal growth factor, has molecular weight, heat sensitivity and biological activity similar to the factor found in bone conditioned medium. Many other biologically active factors have also been extracted from bone cells or demineralized bone by different laboratories. Their actions on bone cells range from chemotactic to mitogenic. These recently discovered bone factors emphasize that there is important regulation of bone metabolism at the local level.

Key words: Bone volume regulation — Skeletal coupling — Skeletal growth factor — Bone derived factors.

The two major functions of bone are to provide mechanical support to the body and to transfer mineral to blood during times of low calcium or phosphate stress. Under normal conditions, the strength of the bone is largely dependent upon its volume. When subjected to a chronic increase in mechanical stress, a bone increases in volume to support the additional load. Hence, the adaptation of bone to changes in loading is necessary to fulfill its function as a structural support. Similarly, the adaptation of bone to changes in serum calcium and phosphate is necessary to fulfill its function as a reservoir of mineral, and this presents a paradox. Use of the mineral reservoir decreases bone volume and therefore decreases mechanical strength. This effect is minimized because the bulk of the mineral reservoir is located at the endosteal surface of the bone where a decrease in bone volume has a smaller effect on mechanical strength than an equal decrease in volume at the periosteal surface would have. Even so, we would expect that any reduction in bone volume due to the use of the mineral reservoir should be temporary and compensated by a subsequent increase in formation, if mechanical strength is to be preserved. But how are these opposing, dual functions of the skeleton controlled?

Although much is left to learn, recent work has provided some insight into bone volume regulation. Two basic mechanisms appear to be operative: systemic regulation by calcium and phosphate regulating hormones, and local regulation, which is the focus of this paper. One of the local mechanisms for bone volume regulation is mechanical stress. There are at least two other processes that probably function locally to regulate bone volume: the coupling of bone formation to resorption, and bone repletion. One example of coupling is as follows. In mild dietary calcium deficiency, serum calcium tends to drop and this is prevented by an increase in serum parathyroid hormone (PTH) which in turn increases serum $1,25(OH)_2D_3$ [1]. Parathyroid hormone increases the tubular reabsorption of calcium and thereby

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produces calcium conservation by the kidney. $1,25(OH)_2D_3$ increases fractional calcium absorption by the gut such that even though the dietary level of calcium may be low, the percentage absorbed is greater than normal. These two systemic hormones act on bone to increase bone resorption, which also tends to correct any serum calcium deficit; however, the increase in bone resorption is subsequently balanced by a localized compensatory increase in bone formation (i.e., coupling) and thus bone mass is preserved while a normal serum calcium is maintained [2, 3]. The coupled increase in bone formation is mediated by an increase in osteoblast number.

If the dietary level of calcium is further depressed, the gut and kidney conservation mechanisms can no longer compensate, and serum calcium falls. This in turn inhibits the coupled increase in bone formation thereby allowing net loss of bone mineral from the bone mineral reservoir to promote serum calcium [4]. Interestingly, osteoblast number is increased during this phase of the response, but osteoblast activity is depressed. Once the severe dietary calcium stress abates and the serum calcium returns to normal, a repair process termed *bone repletion* begins. Bone repletion is characterized by a marked increase in bone formation which continues, though at a declining rate, until the deficit in the bone mineral reservoir is corrected [4]. Recently, bone repletion has been observed *in vitro* [5].

Of the bone volume regulatory mechanisms so far identified, mechanical loading responses and bone repletion are known to be local or inherent to bone. Moreover, our recent work indicates that the coupling of bone formation to resorption is also locally regulated. From a mechanistic standpoint, neither the regulation of bone volume by mechanical stress nor the regulation of bone repletion are well understood. In contrast, recent work has clarified the mechanism of coupling, and the primary focus of the remainder of this paper is on the cellular and chemical basis for bone coupling.

Coupling *in vivo*

One of the first clues to understanding the regulation of bone volume was the demonstration by Harris and Heany that bone formation and resorption are correlated (i.e., coupled) over a wide range of bone turnover rates in humans [6]. We felt that understanding this relationship between bone formation and resorption was essential to the understanding of bone volume regulation, and for this reason, we set out to develop a rat model of coupling *in vivo*. It was known at that time that, in rats, PTH treatment increased bone resorption and that *in vitro* PTH treatment inhibited formation [2]. In contrast, we found that chronic treatment of rats with PTH produced an increase, not a decrease, in endosteal bone formation along with the expected increase in bone resorption [3]. Thyroidectomized rats also responded to PTH by increasing the endosteal formation rate, eliminating calcitonin as a mediator of this action of PTH [5].

To find out whether this intriguing finding of increased bone formation was due to increased PTH or increased bone resorption, we treated rats with high doses of another resorptive agent, vitamin D, which also led to increases in bone formation and resorption [5]. From these results, it appeared that the increase in bone formation was a response to the increase in resorption (i.e., coupling of formation to resorption) rather than to the nature of the resorber used. Also, a lack of correlation between circulating PTH, $1,25(OH)_2D_3$, Ca, P, or Mg and the increase in formation argued against the idea that coupling is regulated systemically by these effectors [3]. Thus, we sought to test the idea that coupling of bone formation to resorption is a locally regulated process inherent to bone.

Coupling *in vitro*

Addition of PTH to embryonic chick tibiae grown in serum-free culture medium produced a highly significant increase in bone formation beginning on day 2, and persisting throughout the remainder of the 12-day experiment. There was also a prolonged increase in bone resorption beginning a few hours after the addition of the hormone and continuing until the end of the culture period. Because the acute action of PTH (i.e., from 1–24 h) was a decrease in bone formation and because the bones were exposed to PTH only during the first 24 h (subsequently rinsed and fed with fresh medium), it seems unlikely that the chronic increase in formation is attributable to a direct action of PTH [7]. This was the first time a coupled increase in bone formation in response to an increase in resorption had been demonstrated *in vitro*. Treatment of embryonic chick tibiae with another resorptive agent, $1,25(OH)_2D_3$ produced similar results [7]. These data strongly suggest that the increase in bone formation was dependent upon resorption (i.e., coupling) rather than a direct response to PTH or $1,25(OH)_2D_3$.

Next we found that conditioned medium (which did not contain PTH), collected from bones that had been stimulated to resorb (by PTH), contained a macromolecular factor that stimulated bone formation (collagen synthesis and mineral deposition)

in vitro [7]. The active principle in the conditioned medium also stimulated proliferation of monolayer cultures of chick calvaria cells [3]. These results suggest that coupling of bone formation is mediated by a local factor (coupling factor) which is released either from bone matrix or perhaps from bone cells.

Isolation of Coupling Factor from Conditioned Medium

The active principle isolated from the conditioned medium stimulated *in vitro* bone formation, as evidenced by the increase in dry weight and collagen synthesis over that found with fresh medium [7]. Partial purification and characterization of this factor from the conditioned medium indicated that it is an acidic protein with an approximate molecular weight of 75,000. In addition, the bone formation stimulatory activity coeluted with a mitogenic activity which stimulated DNA synthesis in monolayer cultures of chick calvaria cells [8]. Subsequent work aimed at finding the source of this active principle led to the discovery of a potent cell growth stimulator in embryonic chick bone extracts and later in adult human bone extracts [3, 9]. The active principle isolated from human bones has molecular weight, heat sensitivity, and biological activity similar to that of the factor isolated from medium conditioned by resorbing bones and hence was initially termed putative coupling factor [3, 9]. However, since the true *in vivo* function of the mitogen isolated from adult human bones has not yet been established, we designated this factor as "human skeletal growth factor" (hSGF).

Skeletal Growth Factor

Properties of hSGF: Chemical and Biological

Skeletal growth factor is a high molecular weight protein isolated from adult bones of human and several other species such as bovine, porcine, rat, and chick [10–12]. Human skeletal growth factor, purified by nondenaturing polyacrylamide gel electrophoresis, was found to have an apparent molecular weight of 83,000 daltons. Its activity is sensitive to trypsin and resistant to inactivation by heat, extremes of pH, collagenase, and reducing agents [10]. It stimulated DNA synthesis several hours after its addition to quiescent cells [11]. Human skeletal growth factor has been found to stimulate ^{3}H-thymidine incorporation in mesodermal cells: cultured chick embryo bone cells and cartilage cells, human bone cells and skin cells, osteosarcoma cells, chick embryo skin cells, and Balb/C 3T3 cells [12]. No stimulation was observed in cultured kidney, muscle, or liver cells isolated from 17 day chick embryos [11]. In addition to stimulating proliferation in cultured cells, hSGF also increased ^{3}H-proline incorporation (determined as hydroxyproline) into collagen in organ-cultured chick embryo tibiae or calvariae [11].

hSGF is Different From Other Known Growth Factors

To find out whether hSGF is distinct from other polypeptide growth factors, we examined the effects on DNA synthesis of known growth factors in combination with hSGF. Mitogenic activities of epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and insulinlike growth factor-I (IGF-I) were determined with and without added hSGF using quiescent Balb/C 3T3 fibroblasts. The effect of hSGF to maximally stimulate ^{3}H-thymidine incorporation into DNA was sevenfold greater than that of EGF, threefold greater than that of IGF-I and PDGF, and 1.4 times greater than that of FGF [12]. Maximal stimulations by these mitogens were significantly elevated by the addition of hSGF, indicating that hSGF stimulates cell division by a rate-limiting mechanism different than that of the above four polypeptide growth factors that were tested. Stimulation of chick embryo calvarial cell proliferation by fetal calf serum at maximally effective concentrations was further enhanced by the addition of hSGF, indicating the hSGF is different from the growth promoting agents present in the serum, including fibronectin and transferin as well as polypeptide growth factors [13].

The ability of certain proteolytic enzymes to initiate DNA synthesis and cell division has been known since 1970 [14]. Studies with protease inhibitors indicated that the activity of the enzyme was essential for the initiation of cell proliferation. To find out whether the growth-promoting activity of hSGF is mediated by proteolytic activity, we determined the activity of hSGF in the presence of protease inhibitors (phenylmethylsulfonyl fluoride and soy bean trypsin inhibitor). The mitogenic activity of hSGF was not inhibited by these inhibitors, indicating that hSGF action is not mediated by a serine active protease [13].

Using a cultured embryonic chick calvarial cell proliferation system, we found that PTH at 0.1 nm concentration can also act as a mitogen by stimu-

Table 1. Bone-derived factors

Property	Growth factor		Osteo inductive factor			Chemotactic factor	
	hSGF	BDGF	BMP	ECMDF	IMOF	Somerman et al. [27]	Mundy & Poser [28]
M.W.	83K	25K	17.5K	14–22K	0.5–10K	60–70K	<5K
Stability							
Heat	+	+				−	
Acid	+	+			+		
Reducing agents	+		−				
Trypsin	−		−	−	−	−	+
Collagenase	+		+	−	+		
Solubility in H_2O	Soluble		Insoluble	Soluble			
Target cells/organ	Bone cells	Calvaria	Mesenchymal cells	Fibroblasts	Mesenchymal cells	Osteoblasts	Monocytes, tumor cells, bone cells
Source	Bone	CM from Calvaria	DBM	DBM	DBM	DBM	NCP from bone matrix

hSGF = human skeletal growth factor, BDGF = bone-derived growth factor, BMP = bone morphogenetic protein, ECMDF = extracellular matrix-derived factor, IMOF = intramembranous osteogeneic factor, + = resistant, − = sensitive, DBM = demineralized bone matrix, NCP = noncollagenous protein (See text for corresponding references.)

lating ^{3}H-thymidine incorporation into DNA [13]. However, the stimulation by hSGF was severalfold greater than that of PTH, and the addition of PTH to a maximally effective concentration of hSGF further increased ^{3}H-thymidine incorporation, suggesting that hSGF is not a PTH-like substance [13].

Table 1 shows that, based on a comparison of chemical and biological properties, hSGF differs from other known bone-derived factors. In summary, studies so far indicate that hSGF is (1) different from other known polypeptide growth factors (EGF, FGF, IGF-I, PDGF); (2) different from other known bone-derived factors; (3) not the major mitogenic activity in the serum; (4) not a PTH-like substance; and (5) not a serine active protease.

Interactions Between hSGF and Calcium-Regulating Hormones

Based on our *in vitro* studies on the effects of PTH and $1,25(OH)_2D_3$ on the mitogenic activity of hSGF, it appears that calcium-regulating hormones may modulate the mitogenic activity of hSGF [13]. Further knowledge of this interaction between systemic hormones and local factors may provide further insight in understanding the regulation of bone volume.

Other Bone-Derived Factors

Investigators from other laboratories have also reported factors isolated from bone that may be involved in regulation of bone metabolism. Bone-derived factors other than hSGF are described in this section.

Bone-Derived Growth Factor

Canalis et al. [15] reported that conditioned medium from fetal calvaria in organ or cell cultures stimulated the incorporation of ^{3}H-thymidine into DNA, and ^{3}H-proline into bone collagen. They attributed this to the presence of nondialyzable and heat stable growth factors in the conditioned medium [15]. Two fractions that were active on bone formation have been obtained upon partial purification of the conditioned medium. One fraction (bone-derived growth factor 1, BDGF1) with a molecular weight of about 10,000 daltons contained stimulatory activity for proline incorporation into collagen, and the other fraction (BDGF2) with an approximate molecular weight of 25–30,000 daltons contained stimulatory activity for the incorporation of ^{3}H-thymidine into DNA. Bone-derived growth factor 1 has been shown to cross-react with antibodies for somatomedins whereas BDGF2 resembles PDGF in its effect on bone cells and molecular weight. These studies reveal that fetal rat calvariae contain at least two factors, one that may be involved in the regulation of DNA synthesis and the other in the regulation of collagen synthesis [16]. Their *in vivo* role, however, in the regulation of bone metabolism remains to be established. Recently, Canalis and Termine have also shown that osteonectin, a major extracellular protein

of bovine bone, increased synthesis of both collagenous and noncollagenous protein without influencing DNA synthesis in 21-day fetal rat calvariae in culture. In addition, a breakdown product of the native osteonectin molecule stimulated DNA synthesis with no effect on protein synthesis [17].

Osteo Inductive Factors

In 1965, Urist discovered that demineralized bone matrix could induce bone formation ectopically [18]. Several papers have been published since then demonstrating the role of extracellular matrix in local bone induction (see ref 19). The laboratories of Urist, Reddi, and recently, Schmidt have been subsequently involved in the purification of the active components from bone matrix that cause osteoinduction [19–21].

Bone Morphogenetic Protein. During the past two decades, Urist et al. have focused their research on osteoinduction—the induction of cartilage and bone formation in mesodermal tissue by implanted bone or components of bone matrix. The ability of demineralized bone matrix to induce bone formation has been attributed to a low molecular weight protein called *bone morphogenetic protein* (BMP). This protein has been isolated from demineralized bone matrix of several different species [19]. There was a dose-dependent increase in the quantity of new bone formed in response to partially purified bovine BMP implanted in the hind quarter muscles of mice [19]. After the implantation of BMP, the following processes have been reported to take place in the implanted site: (1) absorption of implanted matrix; (2) appearance of small, round cells, macrophages, spindle-shaped cells, and connective tissue cells in the implanted site; (3) morphogenesis of the mesenchymal cells to chondro-osteo progenitor cells; (4) cytodifferentiation of progenitor cells to differentiated cell types (chondroblasts, osteoblasts, osteocytes); (5) colonization by bone marrow cells and vascularization of the implanted area. According to Urist et al. [19], the entire process of new bone formation takes about 4–5 weeks after implantation.

Bone morphogenetic protein is different from hSGF in several of its properties, such as molecular weight, solubility, and sensitivity to reducing agents (Table 1). The molecular weight of BMP is reported to be around 17,500 daltons and is a hydrophobic glycoprotein with 20.8% acidic amino acids [19]. Bone morphogenetic protein activity is trypsin labile but resistant to collagenase and cathepsin treatment [22]. It is also sensitive to reducing agents as its osteoinductive capacity is lost in the presence of DTT or β-ME [23]. An osteoinductive glycoprotein with properties similar to BMP has also been isolated from mouse and human osteosarcoma cell lines [19]. The relationship between the osteogenic factor isolated from these cell lines and the BMP isolated from the bone matrix is as yet unknown. The mechanism of action of BMP at a molecular level in initiating the differentiation of mesenchymal type cells into cartilage and bone remains to be established.

Extracellular Matrix Derived Factor. Reddi and Huggins [24] have also shown that cell-free demineralized collagenous bone matrix, when implanted, can induce formation of cartilage, bone, and bone marrow at the site of implantation. In 1979, Rath and Reddi [25] provided *in vivo* evidence for the mitogenic action of collagenous bone matrix. When demineralized bone matrix powder was transplanted subcutaneously, it stimulated the incorporation of ^{3}H-thymidine into an acid precipitable fraction in the vicinity of implanted matrix. Subcutaneous implantation of demineralized bone matrix in allogeneic rats resulted in the local induction of endochondral bone and a characteristic sequence of cellular changes that is much like embryonic cartilage and bone development [24].

Further studies involving the extraction of demineralized bone matrix from tibiae and femora of adult rats and separation of proteins by using gel filtration chromatography revealed that there are two active principles: a chemotactic factor and a mitogen. The mitogenic activity is associated with a protein of molecular weight 22,000 daltons and is thought to be involved in the initiation of osteoinductive response. This mesenchymal cell growth factor, present in the demineralized bone matrix, has been shown to be sensitive to collagenase and trypsin but resistant to chondroitinase [26]. It is not known whether the osteoinductive protein isolated by Sampath and Reddi is related to BMP.

Intramembranous Osteogeneic Factor. Recently, Thielmann et al. [21] have provided evidence for the presence of a factor in bone matrix that induces intramembranous osteogenesis which involves epithelial-mesenchymal interaction. In intramembranous ossification, there is a direct development of bone from mesenchyme. For the extraction of this factor, the diaphyseal bone of pig is defatted (chlo-

roform:methanol), demineralized (7% HCl), and then solubilized with 0.5 M TCA or bacterial collagenase. The factor inducing the intramembranous osteogeneic activity has a molecular weight of less than 10,000 daltons and is not species specific [21].

Bone Chemotactic Factor

It has been suggested that during remodeling and repair of bone tissue, bone cells or their precursor cells are attracted by a chemotactic mechanism to the areas of bone formation. A chemotactic factor with a strong chemotactic activity for osteoblasts was first isolated by Somerman et al. [27] from the guanidine extracts of demineralized bone powder of rat long bones. This protein has a molecular weight of around 60,000–70,000 daltons and is sensitive to heat and trypsin [27].

Recently, Mundy and Poser [28] have demonstrated the chemotactic effect of osteocalcin, the gamma-carboxyglutamic acid containing noncollagenous bone matrix protein. Using the Boyden chamber technique for measuring cell chemotaxis, Mundy and Poser have shown that osteocalcin is a potent chemoattractant for cultured rat osteosarcoma cells (osteoblastlike), breast cancer cells, and monocytes. A synthetic peptide which resembles the breakdown product of osteocalcin also contains chemotactic activity. We still do not know whether chemotactic factor (M.W. 60–70K daltons) isolated by Somerman et al. from demineralized bone matrix is an osteocalcinlike protein. However, these studies provide provocative indirect evidence that proteins released during resorption may be responsible for attracting the osteoblasts and other precursor cells to endosteal bone surface by their chemotactic activity.

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