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Bone Cell Differentiation and Growth Factors

Marshall R. Urist, Robert J. DeLange, G. A. M. Finerman

Summary. Bone morphogenetic protein and bone-derived growth factors are biochemical tools for research on induced cell differentiation and local mechanisms controlling cell proliferation. Bone morphogenetic protein irreversibly induces differentiation of perivascular mesenchymal-type cells into osteoprogenitor cells. Bone-derived growth factors are secreted by and for osteoprogenitor cells and stimulate DNA synthesis. Bone generation and regeneration are attributable to the co-efficiency of bone morphogenetic protein and bone-derived growth factors.

Bone differs from other tissue not only in physiochemical structure but also in its extraordinary capacity for growth, continuous internal remodeling, and regeneration throughout postfetal life, even in long-lived higher vertebrates. How much of this capacity can be accounted for by proliferation of predifferentiated osteoprogenitor cells and how much can be attributed to induced differentiation of mesenchymal-type cells have been challenging questions for a long time. A basic assumption is that regeneration occurs by a combination of the two processes. The process of induced cell differentiation has been observed from measurements of the quantities of bone formed in response to implants of either bone matrix or purified bone morphogenetic protein (BMP) in extraskeletal (*1*) and intraskeletal (*2*) sites. The osteoprogenitor cell proliferation process has been well known for more than a century and is measured in reactions of periosteum and endosteum to injury, diet, vitamins, and hormones. Bone-derived growth factors (BDGF) stimulate osteoprogenitor cells to proliferate in serum-free tissue culture media (*3, 4*). The mechanisms of action of BMP and BDGF are primarily local, but secondary systemic immunologic reactions could have either permissive or depressive effects.

Recent progress in the field, surveyed in this article, suggest that BMP and BDGF are coefficient; BMP initiates the covert stage and BDGF stimulates the overt stage of bone development. The effects of BMP are observed on morphologically unspecialized mesenchymal-type cells either in systems in vitro or in vivo. The action of BDGF is demonstrable only in tissue culture, ostensibly on morphologically differentiated bone cells.

Induced Bone Morphogenesis

The theory of cell differentiation by induction originates in observations on transplants of embryonic tissues and is a main tenet of modern developmental biology. Development begins with a morphogenetic phase and ends with a cytodifferentiation phase (*5*). The morphogenetic phase consists of cell disaggregation, migration, reaggregation, and proliferation. Through interaction of intra- and extracellular influences, cytodifferentiation follows and a mature functional specialized tissue emerges. As cytodifferentiation occurs, pattern formation is established by the positional values of cells in a three-dimensional extracellular coordinate system (*6*). Pattern formation is a difficult concept to explain because it is heritable, encompasses morphogenesis, and is the culmination of manifold physiochemical processes. Present evidence indicates that chondro-osteogenetic gene activation is induced at the onset of the morphogenetic phase of bone development and is regulated by a combination of extra- and intracellular factors.

Previous studies on extracellular matrix factors consisted chiefly of biochemical interpretations of descriptive morphology. The emphasis has been on uncertainties about when the morphologically predifferentiated (protodifferentiated or covert) stage of development begins, if and when an inductive agent is transferred from extracellular matrix to responding cell surfaces, and how alterations in the genome occur. Since alterations ultimately occur at the level of DNA, clear-cut distinctions between ex-

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tra- and intracellular influences ordinarily are impossible.

The most conclusive evidence of induced bone cell differentiation comes not from an experiment on embryonic growth but from one on postfetal cartilage and bone development. This experiment consists of inducing bone formation in implants of allogeneic bone matrix in a muscle pouch in rodents (*7*). When bone matrix demineralized by treatment with 0.6*N* HCl is implanted in a muscle pouch, perivascular mesenchymal cells disaggregate and migrate into the area of the implant, reaggregate, proliferate, and differentiate into cartilage and bone. The quantity of new bone is proportional to the mass of the implanted matrix. The matrix shows cross-species reactivity. The reaction is so consistently reproducible that it is being used for quantitative analysis of the products of normal bone cell differentiation (*8*). Nearly every biochemical measurement of embryonic skeletal tissue either has been or can be made on postfetal matrix-induced bone (*9*).

In the case of postfetal bone formation, the covert or protodifferentiated state (the major obstacle to recognition of induced cell differentiation in embryonic systems) is not a problem, because the BMP alters the activity of a previously stable cell population of adult (not embryonic) tissue in the intact animal. For example, matrix or matrix-derived BMP is implanted in a muscle pouch and, within 5 to 7 days, mesenchymal cells that otherwise would produce only fibrous tissue in the lifetime of the individual differentiate into cartilage and bone. This morphogenetic response, one of the most consistently reproducible effects known to the field of hard tissue biology, is evoked by mesenchymal-type cells with chondro-osteogenetic competence inherited from the cells of embryonic somites and limb buds. Competence is a not-yet-activated state of readiness, a condition of differential gene activation, and the sum of previous embryonic inductions. A competent cell population receives a developmental signal and as a consequence becomes "determined." All cells, both embryonic and postfetal, have some level of determination insofar as no pleuripotent undifferentiated forms have been demonstrated in vertebrate species. Nathanson *et al.* (*10*) found that at specified stages of development not only mesenchymal-type cells but also myoblasts are competent and acquire chondrogenetic determination in response to bone matrix.

BMP has been isolated from the demineralized dentin matrix of the rabbit

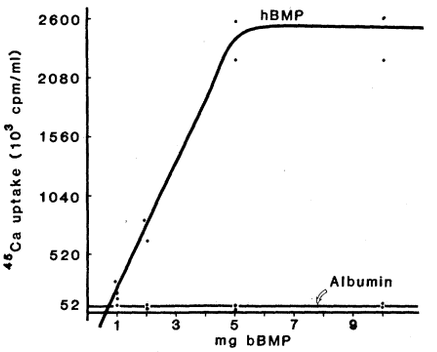

Fig. 1. Quantities of new bone formed in response to graduated doses of purified bovine BMP (*bBMP*) implanted in the hindquarter muscles of the mouse. The graph shows the plateau in the levels of the response to 5 and 10 mg, and the limits of the limb tissues to produce new bone in response to bBMP.

(*11*); from rat (*12*), rabbit (*13*), bovine, and human bone matrix (*1*); and from mouse (*14–16*) and human (*17*) osteosarcoma tissues. Purification of BMP is monitored by implantation in the hindquarter muscles of mice or in skull trephine defects in rats, dogs, and monkeys. When 5- and 10-milligram doses of protein fractions are implanted in muscle, grossly visible bone formation appears at the site of implantation. Doses of less than 1 milligram produce deposits detectable by ^{45}Ca uptake in new bone mineral that are hardly visible in microradiographs. When solubilized in 4*M* guanidine hydrochloride (GuHCl) and reassociated with high molecular weight proteins or with residues of extracted bone matrix, implants (200 micrograms to 1 milligram) are slowly absorbed and produce relatively large new bone deposits. Qualitatively, human BMP (hBMP) and bovine BMP (bBMP) induce identical responses. Quantitatively, bBMP obtained from 1-year-old steers is more active in lower doses than hBMP obtained from 20- to 60-year-old humans, but in any case, in doses up to 5 milligrams, the yield of new bone is directly proportional to the quantity of the implanted BMP (Fig. 1).

Purified BMP induces the same response as bone matrix, but is absorbed by day 7 and sequentially replaced by small round cells, macrophages, amoeboid mesenchymal cells, spindle-shaped cells, and hypertrophied connective tissue cells. By days 14 to 21, a fibrous envelope forms around the reactive connective tissue cells. Inside the envelope, differentiation of chondroid, cartilage, and woven bone occurs. The chondroid and cartilage invariably grow in the avascular interior while the new bone develops on the vascularized exterior of the implanted area. During the interval from day 21 to day 28, the woven bone is remodeled to produce a shell of lamellar bone filled with bone marrow (Fig. 2, a and b).

Bioassays in bone defects suggest that the bone marrow stroma cells are more sensitive to BMP than almost any other known mesenchymal cell population in the body (*18*). Trephine defects in the rat skull, 0.8 centimeter in diameter, too large for spontaneous repair, completely heal within 4 weeks after implantation of BMP (*2*). Chondroid and cartilage tissues normally not found in the cranial vault appear by day 7. Woven bone replaces cartilage by day 14. Lamellar bone and bone marrow replace woven bone by day 28. In response to BMP, both cartilage and bone differentiate from outgrowths of connective tissue from the subcutis and dura as well as from the osteogenetic tissues (for example, bone marrow stroma) of the bony rim. Control implants of bovine albumin and protein subfractions lacking BMP induce formation only of fibrous tissue.

One enzymic and three nonenzymic procedures (*1, 13*) have been devised for extracting BMP from cortical bone. The quantity of BMP relative to the total tissue weight is so small that the large bones of bovine and human species are advantageous for biochemical procedures. Per unit weight, cancellous bone matrix contains less BMP than cortical bone, and is under investigation for synergistic growth factors, inhibitor molecules, and macromolecular carrier substances. (BMP, derived from all known sources, is more completely extractable by GuHCl or an inorganic-organic mixture of calcium chloride and urea than by other solvents.) The BMP is separated from high molecular weight proteins, including gelatin peptides, under dissociative conditions in 4*M* GuHCl and recovered with several other low molecular weight proteins by differential precipitation under associative conditions in 0.25 to 0.5*M* GuHCl. The proteins are again solubilized and fractionated by gel filtration, preparative gel electrophoresis, and hydroxyapatite (HA) affinity chromatography; they are then weighed and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 3).

BMP is very slightly soluble in neutral salt solutions, HCl at *p*H 2, and serum-free culture media. It is completely inactivated under even slightly alkaline conditions; it is also relatively soluble in neutral buffer solutions in the presence of other proteins of a collagenase digest and, provided that sulfhydryl-group enzyme inhibitors are present in the sys-

tem, a high level of biologic activity can be sustained (*13*). Enzyme inhibitors are essential in all chemical extraction systems for BMP because the bone tissue contains endogenous enzymes that attack it (called BMPases) (*19, 20*). The adverse effects of EDTA on BMP have also been accounted for by the action of these enzymes (*21*). The carbohydrate moiety of BMP may not be essential for biologic activity because bone matrix degraded by chondroitinases A, B, and C, amylase, hyaluronidase, and neuriminadase is almost as active as undegraded normal matrix (*21*). However, enzymic degradation experiments have not been performed on purified BMP with a complete battery of carbohydrate-degrading enzymes.

The predominant component of protein fractions with BMP activity has molecular weight of 17,500 (17.5K), with variable quantities of 14K, 24K, and 34K proteins. Each of the last three can be removed without loss of BMP activity. The isolated 17.5K component is more rapidly adsorbed than the 17.5K associated with other proteins and induces a correspondingly lower yield of bone. Differential analysis suggests that the 17.5K putative BMP, consisting of 20.8 percent acidic amino acids, is an acidic protein (Table 1). Antibodies to this 17.5K protein have been produced in mice, for possible radioimmunoassay of BMP (*22*).

The relatively high yields of new bone from partially purified compared to pure 17.5K suggests that the 34K, 24K, and 14K proteins are BMP subunits. Figure 4 illustrates how the effects of BMP could become locally attached to receptors on mesenchymal-type target cell surfaces.

Because induced bone formation is eliminated by dissociation of GuHCl-soluble proteins from rat bone matrix and restored by their reassociation, and because collagen is a substrate for many embryonic tissues developing in culture, Sampath and Reddi (*23*) consider collagen essential for osteoinduction. Others contend that deposition of new bone is not limited to bone matrix surfaces, but also arises within perivascular connective tissues at some distance from bone collagen (*7*). More convincingly, bone formation is induced by implants of BMP in the absence of preexisting bone collagen (*14, 15*). The protein dissociates from the bone matrix and collagen according to the laws of diffusion of small molecules (*24*). The dissociated BMP is transferred across as many as five membranes (a distance of 450 micrometers) through pores as small as 25 nanometers in diameter, to induce bone formation on the outer surface of a Millipore chamber that is implanted in muscle. Because electron micrographs show no collagen fibrils within the pores, we can exclude collagen as a carrier or as an essential part of BMP structure. Furthermore, autolytic digestion of bone cells and limited tryptic degradation of matrix noncollagenous proteins, remove all BMP activity from bone matrix without any loss of bone collagen EM (electron micrography) structure (*25*). Thus, if transmission requires a carrier, it is not collagen, but some noncollagenous bone matrix protein associated with collagen.

Table 1. Amino acid analysis of the putative 17.5K BMP and associated proteins.

Amino acid	Amount (moles per 100 residues)		
	14K	17.5K	24K
Aspartic acid	15.9	10.2	14.3
Threonine	3.8	4.0	6.3
Serine	8.6	11.4	9.5
Glutamic acid	17.9	10.7	8.8
Proline	4.6	7.4	6.0
Glycine	3.9	8.9	9.4
Alanine	12.0	6.6	7.7
Cysteine	1.1	1.9	0
Valine	4.4	5.7	4.8
Methionine	1.4	1.5	1.9
Isoleucine	4.0	4.6	3.9
Leucine	5.8	7.6	6.9
Tyrosine	1.4	4.1	5.8
Phenylalanine	3.7	4.7	5.4
Histidine	1.2	1.7	3.7
Lysine	4.6	4.0	2.9
Arginine	5.7	5.0	2.7

Physiopathologic Observations on BMP in Bone Matrix

In view of the following considerations BMP is considered to be a physiologic entity. In rats, BMP is growth hormone–dependent and declines in quantity in bone matrix in the course of aging (*26*). In rickets, a disorder characterized by failure of matrix calcification, BMP activity is low. In scurvy, a disorder deranging collagen synthesis, both BMP activity and matrix calcification are unaffected (*20*). Thus, it is the organic matrix of bone, regardless of whether it had undergone calcification, that induces a new bone formation. In lathyrism, a derangement of lysyl oxidase activity, collagen lysino-aldehydic cross-link formation, and other structural abnormalities, bone matrix is also deficient in BMP activity (*27*). The BMP deficiency retards bone cell differentiation and accounts for the failure of fractures to heal in lathyritic animals. Studies on BMP in normal and abnormal autopsy subjects suggest that there may be a relation of the incidence of hip fractures or osteoporosis (or both) in postmenopausal women to BMP deficiency (*28*).

Osteosarcoma glycoprotein-induced bone formation. An osteoinductive gly-

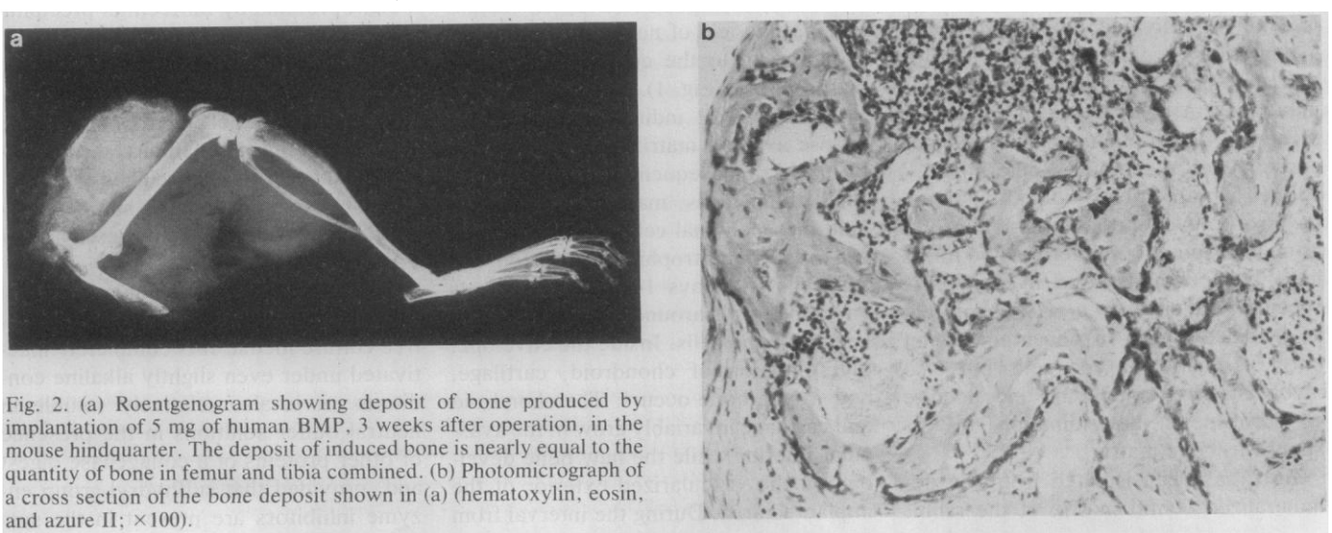

Fig. 2. (a) Roentgenogram showing deposit of bone produced by implantation of 5 mg of human BMP, 3 weeks after operation, in the mouse hindquarter. The deposit of induced bone is nearly equal to the quantity of bone in femur and tibia combined. (b) Photomicrograph of a cross section of the bone deposit shown in (a) (hematoxylin, eosin, and azure II; ×100).

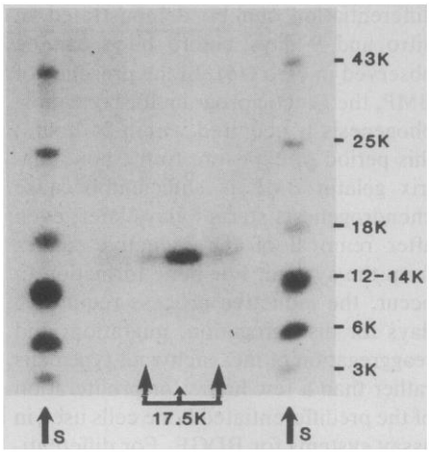

Fig. 3. Electrophoresis (sodium dodecyl sulfate slab, polyacrylamide gel) pattern of a purified BMP with a molecular weight of 17.5K, calculated by plotting the negative logarithm of the relative mobility against the standard proteins (Bethesda Research Laboratories) of known molecular weights.

coprotein derived from mouse and human osteosarcoma cells is named "osteogenic factor" by Amitani and Nakata (*14*) and Takaoka *et al.* (*15*), and identified as osteosarcoma BMP by Hanamura *et al.* (*16*) and Bauer and Urist (*17*). The Dunn osteosarcoma contains more BMP activity (0.1 mg per gram) than is extractable from the entire skeletons of about 100 mice. In mice, Hanamura *et al.* (*16*) found osteosarcoma BMP in protein fractions with molecular weights of 12.5K to 30K while Takaoka and associates (*15*) independently isolated a 22K protein. If the molecular weight of a completely purified protein should prove to be in the range of 22K to 30K, which is significantly higher than that of BMP from bone matrix (approximately 17.5K), osteosarcoma cells may prove to be a source of a precursor of BMP, proBMP. The locus of osteosarcoma BMP appears to be on cell surfaces in that trypsin-disaggregated osteosarcoma cells lose BMP activity and then regenerate the activity in continuous culture. Collagenase-disaggregated cells retain activity and in so doing lend support to the concept of BMP as a trypsin-labile collagenase-resistant noncollagenous protein (*29*). This and comparable observations on bone matrix refute the idea that bone collagen is the BMP and support the more likely possibility that BMP is a is a noncollagenous membrane protein (*13*).

Epithelium-induced bone formation. Transplants of (i) transitional epithelium beneath abdominal fascia in dogs and guinea pigs by Huggins (*30*), (ii) HeLa and amnion FL cells in muscle in mice by Anderson and associates (*31*), or (iii) more than ten different established cell lines in various tissues in mice by Włodarski *et al.* (*32*) induce differentiation of connective tissue cells into cartilage and bone. These observations on postfetal osteogenesis are comparable to observations on embryonic osteogenesis by Hall (*33*), who concludes that initiation, if and when it occurrs, resides not in the cells forming the bone but in adjacent tissues with which they interact. Whether epithelial cells secrete a BMP, or indirectly induce mesenchymal-type cells to synthesize a BMP are unanswered questions. Huggins (*34*) postulates a solid-state physicochemical alteration of cell surfaces producing phenotypic transformation. Observations on osteogenesis induced by implants of lyophilized urinary bladder or placenta in rabbits (*35*) suggest that the biochemical basis for epithelium-induced osteogenesis should be investigated by chemical extraction of epithelial tissues with various solvents used for membrane and nucleoproteins.

The correlation of osteoinductive activity with agglutination of epithelial cells by concanavalin A (Con A) (*31, 32*) is circumstantial evidence of a tissue-specific membrane glycoprotein with BMP activity. With relatively few exceptions, cell lines without osteoinductive activity are Con A insensitive. A BMP fraction, isolated from rabbit bone by means of carbohydrate recognition and hydrophobic interaction (*13*), is direct evidence of an osteoinductive glycoprotein.

Chondro-osteogenetic DNA

Transplants of vaccinia-transformed fibroblasts differentiate into bone cells (*36*). This may be viewed as circumstantial evidence of a chondro-osteogenetic DNA developing in response to an extracellular agent. Convincing evidence that BMP-induced differentiation is a manifestation of differentiation of DNA, comes from experiments with bromodeoxyuridine (BrdU), a thymidine analog (*37*). When BrdU is incorporated into mesenchymal cell DNA, cartilage development is inhibited. If BrdU is incorporated into precartilage mesenchymal cell DNA, inhibition is irreversible; if incorporated into chondroblast or chondrocyte DNA, inhibition of chondrogenesis is reversible. This blockage of BMP-induced cell differentiation is produced by incorporation of BrdU into the genome. Although the specific chemical modifications influencing cell differentiation are not well understood, BrdU appears to modify repressor DNA interactions and become preferentially incorporated into a late moderately repetitive region of DNA and not just indiscriminantly attached to thymidine (*38*).

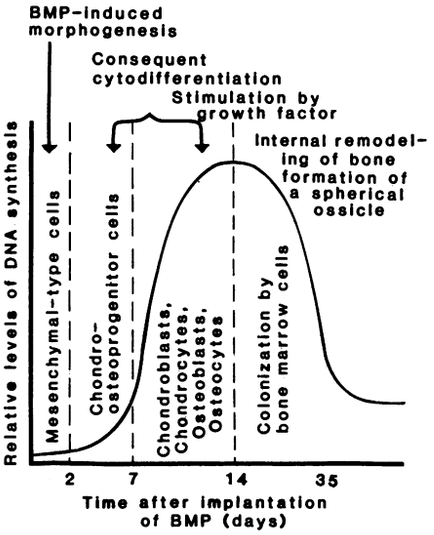

Fig. 4. Relation of bone morphogenesis to cytodifferentiation, expressed as DNA synthesis after implantation of BMP. The target cells for BMP are predifferentiated mesenchymal cells. Bone-derived growth factors are secreted by and for postdifferentiated bone cells, metabolic activity, and therefore functional in the cytodifferentiation phase of development. The curves described by the relative levels of DNA synthesis suggest that the Zwilling (*5*) theory of embryonic organ development is applicable to postfetal bone development. BMP initiates repetition of the embryonic process of bone development by mesenchymal-type perivascular connective tissue cells in postfetal life.

Another DNA probe, 5-azacytidine (AZC), an analog of cytidine used by Jones and Taylor (*39*), promotes, rather than inhibits, differentiation of 3T3 mesenchymal cells into cartilage. It is not known whether AZC produces despecialization (sometimes referred to as dedifferentiation) to a state more pleuripotent than the 3T3 cell, or whether it induces differentiation directly (phenotypic transformation). Whatever the mechanisms of action may be, DNA probes are valuable for research on BMP-induced cell differentiation. A bone morphogenetic process culminating in the formation of a permanent sphere-shaped ossicle, complete with a cortex and marrow cavity, involves substitution of an entire developmental program, not only one or two gene products. Such programs would be written in repetitive nucleotide sequences that are transcribed according to cell type specific patterns. Multiple transcriptions, constituting a chondro-osteogenetic pattern, may be instituted by exposure of competent mesenchymal-type cells to BMP.

Bone-Derived Growth Factors

Growth factors are growth-stimulating substances that are not nutrients. Metabolic substrates, cofactors, vitamins, amino acids, and minerals are classified as nutrients rather than growth stimulants. A number of hormones, such as insulin, growth hormone, somatomedin, and urogastrone, are included among growth factors (*40*). Since the predominant effect is local, Sporn and Todaro (*41*) suggest that growth factors constitute a paracrine-autocrine system. The paracrine factor is transferred from the cell of origin to an adjacent cell population while an autocrine factor is transferred back to the cell itself. Elaborated in very low concentrations, paracrine-autocrine growth factors stimulate DNA synthesis by a specialized cell population. Although some of these factors are produced by more than one organ system, they generally can be classified according to source as fibroblast growth factors (FGF), epidermal growth factors (EGF), platelet-derived growth factors (PGF), and nerve growth factors (NGF). In general, growth factors are polypeptides. Systemic effects, observed with one growth factor (EGF), have not yet been reported with BDGF. The definitive work on EGF, FGF, and other factors has been reviewed by Gospodarowicz and Moran (*42*).

Present knowledge of BDGF emerged from observations on cell proliferation in conditioned media. For example, culture media that was conditioned with trypsin-labile, heat-sensitive, and mercaptoethanol-inactivated substances secreted by specialized cells of a previous cell generation enhanced the growth of the succeeding generation of cells (*43*). The earliest experiments on conditioned media were performed in media supplemented with 10 percent fetal calf serum. At the present time, in experiments on the enhancement of growth by factors secreted by bone cells, serum is omitted from the culture medium; this omission is almost a prerequisite for research on growth factors, as demonstrated by observations on BDGF (*3, 4, 44*). These observations consist of measurements of DNA synthesis by incorporation of ^{3}H-labeled thymidine into embryonic progenitor cells in vitro (*45*) or by activation of ornithine decarboxylase (*9*) over a period of 1 to 2 hours. Incorporation of ^{3}H-labeled proline into type I bone collagen or ^{35}S into proteoglycans can be measured at the same time on the same specimen.

Baylink *et al.* (*4*) described a skeletal growth factor (SGF) and designated it "coupling factor." The local factor regulating the replacement of bone lost by resorption in the normal course of bone remodeling is a hypothetical coupling factor. However, recruitment of mesenchymal cells for an osteogenetic pathway of development and stimulation of osteogenetic progenitor cells are two separate processes. Bone resorption is controlled by still another process, the formation of osteoclasts from blood-borne bone marrow–derived monocytes. The number of osteoprogenitor cells in the adult bone seems too small to replace all of the large volume of bone normally lost in the process of aging of the skeleton. A feedback mechanism of recruitment of new osteoprogenitor cells from the mesenchymal cell pool by BMP plus stimulation of mitosis by BDGF may be necessary to sustain the bone mass on long-lived animals. Whereas a growth factor requirement is fulfilled for many tissues, the recruitment of new cells by a morphogenetic mechanism occurs only in bone tissue.

Table 2. Relative proportions on noncollagenous proteins and collagen in bovine cortical bone. Immunoglobulins IgA, IgD, IgE, IgM, α-acid–glycoprotein, transferrin, and α-antitrypsin are minor or trace components (*44*).

Constituent	Percent (by weight)
Collagen	88.00
Sialoprotein	1.0
Phosphoprotein	0.2
Proteoglycans	1.0
Proteolipids	0.3
GLA-containing protein	1.5
Albumin	0.3
α_2-HS-Glycoprotein	0.4
Lipids	0.4
Peptides	0.8
Structural glycoprotein	1.0
Osteonectin	2.5
BMP	0.1
Unaccounted for	2.0

The possibility of contamination of BMP with BDGF is unlikely because the starting material for preparation of BMP contains no BDGF. The starting material is insoluble bone matrix gelatin from which BDGF is partially removed by demineralization of the matrix with HCl and completely removed by extraction of soluble noncollagenous proteins with calcium chloride and EDTA (*45*). When bone matrix gelatin is used for a substratum for mesenchymal cell outgrowths of muscle, the morphogenetic process can be followed by monitoring hyaluronate accumulation, hyaluronidase activity, and incorporation of ^{3}H-labeled thymidine into DNA, all of which appear within 24 to 48 hours, even though it is at least 6 days before overt cartilage tissue differentiation can be demonstrated in vitro and 9 days before bone can be observed in vivo (*46*). In the presence of BMP, the genetic program for bone morphogenesis is acquired within 24 hours; this period of exposure to the bone matrix gelatin BMP is sufficient because chondrogenesis starts 6 days later, even after removal of the inductive matrix from the system. For bone formation to occur, the inductive process requires 6 days for disaggregation, migration, and reaggregation of mesenchymal-type cells rather than a few hours for proliferation of the predifferentiated bone cells used in assay systems for BDGF. For differentiation of postfetal mesenchymal-type cells into osteoprogenitor cells, the culture media available at present and the variability of avascular conditions in vitro are less than adequate.

On the basis of electron-micrographic evidence of calcifying tissue, three research groups (*47*) demonstrated that bone cells undergo differentiation in cultures of embryonic axial mesenchyme in the absence of exogenous BMP or BDGF. Accordingly there should not be a requirement for exogenous BMP or BDGF by embryonic cartilage organ cultures that grow and begin to develop collars of perosteal new bone in vitro (*48*), supported possibly by synthesis of endogenous growth factors. Postfetal organ cultures of rat calvarium also produce new bone without exogenous growth factors. On prolonged cultivation the explanted old bone is resorbed while the growing new cells differentiate into cartilage. The conditions in vitro, which are adequate for chondrogenesis, provide a barely adequate environment for osteogenesis.

Interpretations and Perspectives

Almost as many different substances are found in bone matrix as are found in blood and other connective tissues of the body. The major components, including some recently discovered noncollagenous proteins, such as α_2HS (*49*), osteocalcein or bone γ-carboxyglutamic acid–rich protein (*50*), osteonectin (*51*), and BMP (*1*), are shown in Table 2. Proteoglycan is soluble in EDTA, and proteolipid is soluble in a mixture of chloroform and methanol. Both are extractable from bone matrix without loss of BMP activity. Phosphoprotein, the component high in phosphoserine residues, is also soluble in EDTA; BMP is not a phosphoprotein because it lacks phosphoserine. Like BMP α_2HS-glycoprotein accumulates in bone matrix, but it has a molecu-

lar weight of 50K, more than twice that of BMP. Osteonectin is soluble in 4*M* GuHCl, lacks BMP activity, and has a molecular weight of 32K, almost twice that of BMP. Structural glycoprotein (*52*) is soluble in 6*M* urea only after mercaptoethanol reduction, while BMP does not require reduction for solubilization. Because mercaptoethanol reduction eliminates BMP activity (*53*), a disulfide-bonded structure may be essential for biologic activity of either BMP or one of its carrier molecules. The molecular weight of BDGF is lower (< 10K) while that of SGF is higher (> 80K), than that of BMP (17.5K).

Neither BDGF nor SGF are known to reprogram the genome of mesenchymal-type cells or derepress genes to produce chondro-osteogenetic DNA. When chondro-osteogenetic DNA is activated by BMP, mesenchymal-type cells (i) differentiate into cartilage and bone, (ii) transmit the genetic program of the induced cells to their progeny, (iii) integrate with bone cells in the process of internal remodeling, (iv) enter a resting stage with the development of a complete ossicle with a cortex of lamellar bone and a marrow cavity. In contrast, BDGF and SGF share with other growth factors the character of unspecific action on specific target cell populations; EGF can accelerate DNA synthesis even by embryonic bone cells; FGF stimulates growth of chondrocytes; a cartilage-derived growth factor and BDGF have in common somatomedin-like properties, as determined by simultaneous increases in incorporation of ^{3}H-labeled thymidine and $^{35}SO_4$ (*3*, *4*).

Purified BMP retains biologic activity after denaturation in 6*M* urea or 4*M* GuHCl and renaturation in physiologic media in vivo. In contrast, BDGF are irreversibly inactivated by urea; they are readily soluble in culture media and assayed in quantities as little as 0.3 μg/ml but not as yet applied to systems in vivo. Preparations of BMP that are only slightly soluble in culture media become completely soluble in body fluids possibly at 37°C. In vivo, BMP is soluble enough to diffuse as many as five membranes, each 125 μm in thickness, with a pore size of 0.45 μm, or two such membranes with pore sizes of only 25 nm.

Implants of bone matrix are mitogenic, promoting tissue proliferation in vitro (*11*) as consistently as in vivo (*9*). In vitro, development does not extend beyond the stage of chondrogenesis. In vivo, chondrogenesis extends into osteogenesis and bone development is induced with equal consistency in either skeletal or extraskeletal sites. BMP stimulates mitotic activity of muscle-derived mesenchymal cells 5 days before the appearance of osteoprogenitor cells with the potential to produce BDGF. The coefficient relationship between BMP and BDGF is illustrated in Fig. 4.

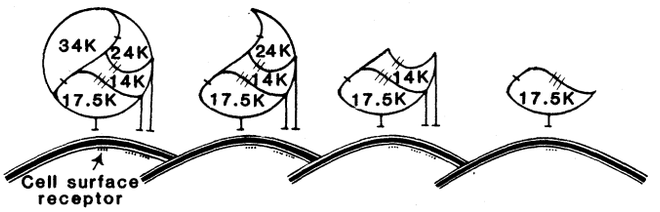

Fig. 5. Representation of BMP and the other three low molecular weight components isolated from bone matrix gelatin. The bond between 24K and 34K components is severed by 4*M* GuHCl (guanidine hydrochloride). The 24K component is isolated by differential precipitation in 0.5*M* GuHCl. The bond between BMP and the 34K component is severed by extraction with Triton X-100. The 17.5K and 14K proteins are dissociated in 4*M* GuHCl; the 17.5K component is isolated by gel filtration and preparative gel electrophoresis.

Differentiation is a process integrated with supracellular controls, physiologic processes, and organismal functions, including positive and negative feedback reactions, growth homeostasis, and the like. The mechanism of cell differentiation under the influence of either epithelial cell lines, or bone matrix, or BMP is not known. Huggins (*30*) postulated a fibroblast transforming factor (FTF) altering cell surface electrical charge, inducing phenotypic transformation, initiating a cascade of cell-cell interactions, and instigating chondrogenesis, osteogenesis, and myelogenesis, respectively. Hypothetically, the binding of a protein with BMP activity to membrane receptors on mesenchymal cell surfaces would produce such alterations in the net charge as may activate a gene regulator molecule, and induce differentiation of chondro-osteogenetic DNA (Fig. 5).

Tangible information on the loci and mechanisms of action of BMP and BDGF in relation to cell surface glycoproteins may be expected to emerge when antibodies to purified BMP and immunofluorescence microscopy become available. Investigations on interactions with carrier and inhibitor proteins will also become possible when purified BMP and BDGF are available in adequate supply. Unexpected as it may seem, bone, the hallmark of vertebrate species, through BMP provides a new approach to the unsolved problem of cell differentiation.

Conclusions

Bone is the only tissue in the body of higher vertebrates to differentiate continuously, remodel internally, and regenerate completely after injury. Whether this capacity can be attributed to BMP and how its activity is supplemented by recently discovered BDGF are questions of both fundamental and clinical significance. BMP induces differentiation of mesenchymal-type perivascular cells into cartilage and bone in either extraskeletal sites or bone defects. The BDGF's, secreted into the media of bone cells in culture, are hydrophilic proteins or protein aggregates that stimulate DNA synthesis, proline transformation into hydroxyproline, uptake of sulfate, and other metabolic processes. BMP-induced development is irreversible, while BDGF growth stimulation is reversible and comparable overall to the effects of somatomedin.

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