Initiation and enhancement of bone formation

A review

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Knowledge of some of the fundamental biochemical factors that may influence the initiation and continued growth of bone-forming cell lines is presented. The discussion is limited to those factors shown experimentally to be present locally in bone tissue and synthesized in the environment of bone-forming cells. The current state of knowledge of basic research findings on osteogenic factors is given in detail. Cooperative actions of these locally produced and systemic factors are the primary stimuli that result in increased bone growth and volume.

Initiation and enhancement of bone formation

All aspects of development of an organism and the induction of tissues are dependent upon molecular interactions (Ham and Vermett 1980); and the generation of the shape and form, or morphogenesis, of bone tissue is no exception. How a particular cell assumes its characteristic shape and phenotype are central issues in biological research. Over the past 20 years, we have learned more about the molecules that participate in these processes in bone tissue; and research activity in the field of bone morphogenesis has accelerated markedly over the past 7 years. The current state of knowledge of the initiation of bone formation and, in part, the subsequent enhancement of bone growth will be considered here.

Mechanisms that control bone growth are complex processes and include the actions of systemic and locally produced factors. Not all aspects of the many factors that participate in the processes of bone growth will be discussed; treatment will be limited to those growth factors produced locally in the tissues (Table 1). Many of these growth factors have been discovered by the modern techniques of cell biology, and their general physiologic effects are being investigated intensely at the present time (Stoker 1985). They are important regulators of gene expression, and are crucial messengers between cells. Some have general cellular and tissue effects, whereas others seem to be more specific and selective for bone cells. Many are involved in bone morphogenesis during embryonic development, and indeed the first embryonic morphogen has recently been identified (Thaller and Eichele 1987, Slack 1987), but this topic is beyond the scope of this review. Here, some of the basic aspects of the development of bone in the postnatal organism will be considered particularly with reference to the initial differentiation of the osteoblastic phenotype.

Table 1. Some factors affecting bone growth

Local growth factors	*Systemic growth factors*
Bone morphogenetic protein	Epidermal growth factor
Skeletal growth factor	Fibroblast growth factor
Transforming growth factor-ß	Platelet-derived growth factor
Bone-derived growth factor	Insulin-like growth factors (somatomedins)
($ß_2$ microglobulin)	
Cartilage-derived factor	
Platelet-derived growth factor	*Local environment*
Hormones, vitamins	Extracellular matrix components
Growth hormone	Plasma proteins
Insulin	Blood flow and nutrition
Glucocorticoids	
Thyroid hormones	*Other factors*
Parathyroid hormone	Electrical
Calcitonin	Mechanical
Prostaglandins	Gravitational
Vitamin D, A, K, etc.	Viruses
	Age

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Lineage of osteoblasts. Which cells give rise to the osteoblastic lineage in the postfetal skeleton? They are obviously cells residing at or near bone surfaces as bone grows by accretion. Proliferating cells must be present in the adjacent marrow tissue or periosteal connective tissue, and some evidence suggests that they are present as a continuum throughout bone spaces. Certain of these cells on or near to bone surfaces exist as preosteoblasts, and there are indications that these are derived from specific stem cells (Friedenstein 1973). The latter are uncharacterized except, by definition, for their potential for self-renewal and for their capacity to produce descendants that differentiate into all types of maturing progeny characteristic of the cell line (Hendry 1985) terminating in osteocytes in this case. Stem cells appear to be present in all regenerating populations; and bone is probably no exception, although this has not been proved conclusively. Current dogma states that these bone-tissue stem or generating cells are equivalent to certain cells of the marrow stromal system present in bone marrow (Owen 1985). In general terms, in any tissue, stroma refers to the supporting tissue in any organ that supports the cells that perform the specific functions, the parenchyma.

Stromal systems

To explain the nature of this marrow stromal system, we must consider briefly the morphology of marrow. The framework, or stroma, of the marrow is a connective tissue and comprises cells that participate in the maintenance and structural support of the hemopoietic cells of the marrow. These cells encourage the development of hemopoiesis and are said to make up the hemopoietic inductive microenvironment (Allen 1981). There is apparently a special relationship between bone and marrow, for wherever true bone is formed, irrespective of the initiating mechanism, it generally leads to new hemopoietic marrow (Trueta 1968, Urist et al. 1969). Connective tissues provide the stroma of almost all the tissues, but the nature of marrow connective tissue appears to provide a special site for hemopoiesis to occur. From the standpoint of the hematologist, the stroma of marrow consists of all the cells, other than the hemopoietic cells, that may actively support hemopoiesis. Indeed, development of cell lineages within the hemopoietic system occurs in association with certain of these discrete stromal elements (Weiss 1981). So macrophages, adipocytes, endothelial cells, reticular and fibroblastic cells are all subsumed under the term marrow stroma (Dexter 1982). Yet, these cells have diverse origins.

From the point of view of bone-cell origin, however, the marrow stromal system (Figure 1) has been given a more restricted definition to include only the network that is made up of fibroblastic cells, reticular cells, adipocytes, and osteogenic cells (Owen 1985). That the bone-forming cells can originate from cells within the marrow system is beyond question. Postnatally, these are totally distinct and histogenetically unrelated to the hemopoietic and lymphopoietic marrow cell lines (Owen 1978, 1980). By elimination and reduction, they are part of the marrow stromal system, but the above important differences in definition must be remembered. If this is not appreciated, the term "marrow stromal stem cell" may be thought to imply that such a cell may give rise to all the components of the marrow stroma. This is clearly not the case. Other components of the hemopoietic marrow stroma, for example, endothelial cells, appear distinct from the hemopoietic and fibroblastic cell lines; and macrophages are actually of hemopoietic origin (Le Douarin et al. 1975, Le Douarin 1979, Wilson 1983, Sahebekhtari and Tavassoli 1978). Thus, there may be a confusion of terminology if the adjective "stromal" is not qualified. Further, osteocytes develop in the embryo before any bone marrow system; and the slightly more specific name "stromal fibroblastic system" will be used here to emphasize the different origins of the osteoblastic cells from the hemopoietic systems in marrow. Because components of stromal fibroblastic systems; are present in most tissues throughout the body, the term will assist clearer interpretation of ectopic ossification.

A stromal stem cell compartment – which includes pluripotent cells widespread in the body, together with specific stem cells – and committed progenitors for each organ have been proposed (Owen 1985). This is still largely a matter of speculation and is complicated by the presence of fibroblastic precursor cells in the blood circulation (Maximow 1928, Luria et al. 1971, Piersma et al.

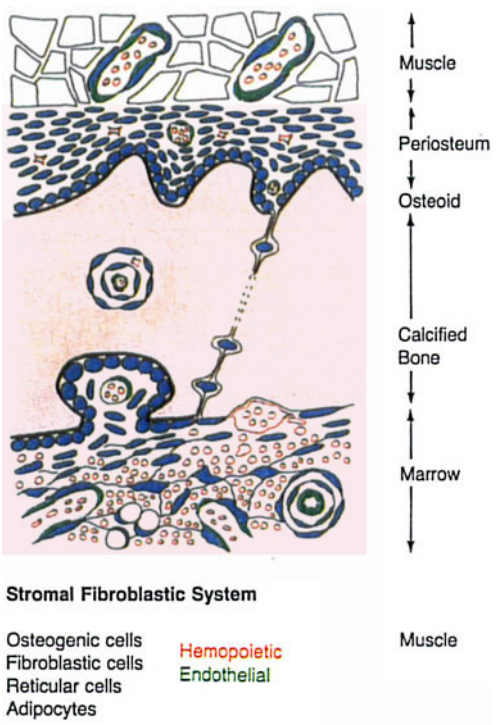

Figure 1. Diagram illustrating the locations of some of the histogenetically distinct cell lines present at bone surfaces. The stromal fibroblastic system (blue) includes osteogenic, fibroblastic, reticular, and adipocytic cells. Cells included in the hemopoietic system (red), endothelial system (green), and skeletal muscle (black) are of distinct origins from those of the stromal fibroblastic system and from each other in the postnatal animal.

1985). However, fibroblastic stromal cells from certain organs, including marrow, do appear to express different antigenic markers that may be related to differences in functional requirements for each organ (Piersma et al. 1985). By analogy with other systems, and particularly with the hemopoietic system about which most is known, a diagram of possible lineages of the cells can be constructed (Figure 2).

Osteogenic progenitor cells

Enhanced normal bone formation may therefore be considered to be caused by a stimulation of commitment of the stem cells and their progeny, which results in cell proliferation and differentiation. The basis of Friedenstein's definition of determined osteogenic progenitor cells (DOPC) is that these presumptive stem cells on bone surfaces are already committed towards the osteogenic direction (Friedenstein 1973). When these cells are removed mechanically with bone marrow and when transplanted heterotopically, they differentiate spontaneously into bone (Patt and Maloney 1972, Tavassoli and Crosby 1968, Friedenstein et al. 1986,). Similar cells probably reconstitute the medullary cavity following injury and ablation (Brånemark et al. 1964, Maloney and Patt 1969, Patt and Maloney 1975).

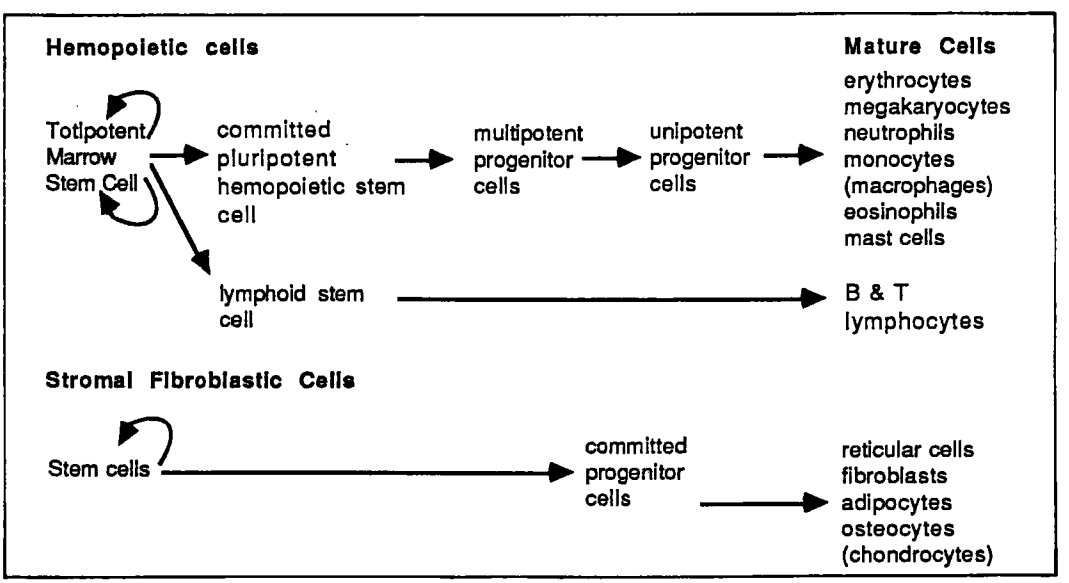

Figure 2. Possible cell lines in the differentiation of the hemopoietic and stromal fibroblastic systems of bone marrow.

The bone-forming cells that are concentrated at bone surfaces can be grown from total bone marrow as an adherent fibroblastic layer in vitro derived from colonies, each generated from a single cell (Friedenstein 1976). When transplanted in an isolated environment in diffusion chambers in vivo, they can form calcified tissue. This tissue, although avascular, resembles the skeletal osseous tissue by both light and electron microscopic evaluation (Ashton et al. 1980, Bab et al. 1984). Not all of these colonies grown in vitro can give rise to bone as Friedenstein (1980) has shown; but a significant proportion, about one third, do.

Ectopic bone formation

It is apparent that cells located in sites far away from bone surfaces, in extraskeletal sites, also have the capacity for true bone formation (Connors 1983, Smith and Triffitt, 1986). The differentiation into bone tissue of unspecialized mesenchymal cell populations in these sites is initiated by a process termed *bone induction*. Huggins (1930, 1931) demonstrated bone induction in classical experiments more than 50 years ago by transplantation of living transitional urinary epithelium into various connective tissues of dogs and rabbits. Subsequently, other living epithelial cells were found to have similar properties (Anderson 1976, Wlodarski 1969). A significant physiologic finding was that normal mouse bone synthesized and secreted a bone-inducing factor (Goldhaber 1961); later studies revealed a similar material in certain mouse and human osteosarcomas (Hanamura et al. 1980, Amitani and Nakata 1974, Heiple et al. 1968, Bauer and Urist 1981). That devitalized bone tissue contained an osteoinductive factor was first shown by Urist (1965), and he named this bone morphogenetic protein, or BMP.

Biochemical isolation of bone-inductive agents. There is now a great deal of research effort being directed to identifying and characterizing the osteoinductive agents present in bone tissue using, as starting materials, demineralized normal or osteosarcomatous bone. Identification of such a factor(s) would allow basic studies on osteogenic induction and osteogenesis and an assessment of its (their) action on mechanisms important in fracture healing and normal and abnormal bone growth.

Since 1978, the techniques for producing a soluble fraction of bone tissue that contains osteoinductive activity have been available (Urist and Mikulski 1979). Biochemical procedures have therefore been applied to the purification of the factor; and since this time, many other solubilizers have been found (Urist and Mikulski 1979, Urist et al. 1981, 1982, Sampath and Reddi 1981). Guanidinium solutions with highly dissociative properties have been used mainly, and they solubilize all detectable osteoinductive agents from bone matrix. By chromatographic procedures, it has been shown that the osteoinductive agents from every source are found in the low molecular weight, noncollagenous protein fractions. The complete chemical characteristics of the osteoinductive agents have not been identified, or if they have, they are closely guarded commercial secrets at the moment. It has been claimed that BMP has been isolated in pure form. Human BMP and bovine BMP are said to have molecular weights of about 18,000 and to have the characteristics of acidic proteins (Urist et al. 1983, Urist et al. 1984, Urist et al. 1986); this contrasts with the basic nature of osteosarcoma-derived BMP, which has an isoelectric point of about 9, although the molecular weights are all similar (Takaoka 1987). The techniques of polyacrylamide gel electrophoresis used to monitor protein purity are not sensitive enough to eliminate the possibility that a minor undetected constituent (or constituents) confers biological activity in the assays. Monoclonal antibodies to the major protein in the purified bovine fractions with BMP activity have been used to measure levels in plasma in normal patients and those with a variety of bone diseases (Urist et al. 1985, Urist and Hudak 1984). This immunoassay obviously measures serum differences in the clinical conditions, but full characterization of the antibody has not been reported.

Assays for inductive activity. A major reason for the difficulties in purification of the osteoinductive agent is the lengthy, problematic, and inconsistent biological assay procedures involved. The earliest assays for bone induction involved surgical implants of relatively large amounts (1–20 mg)

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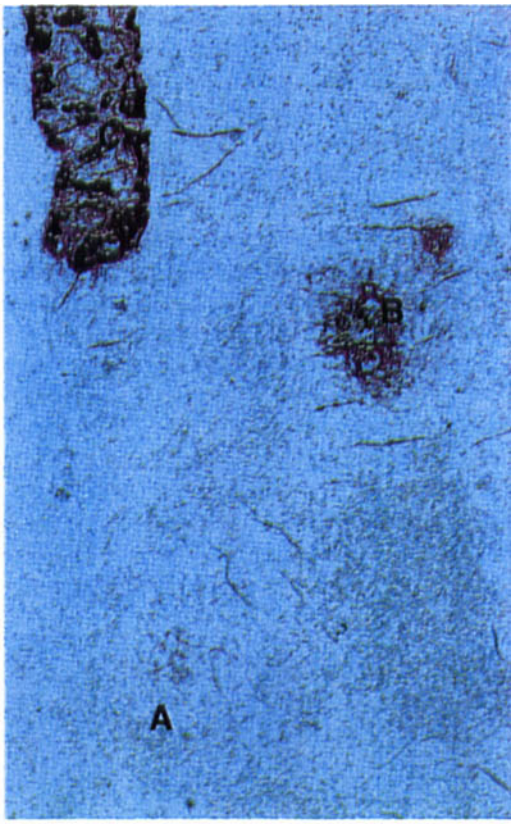

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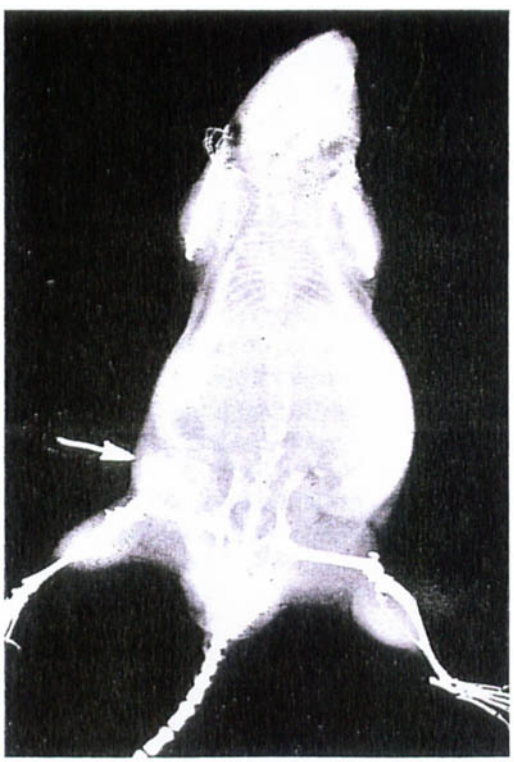

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Figure 3. A. Trabecular bone formed on the outside of a diffusion chamber containing a soluble extract of rabbit bone matrix and bounded by cellulose acetate membranes. The diffusion chamber was recovered 21 days after intramuscular implantation into allogeneic rabbits and prepared for histochemical staining with Von Kossa (black) and for alkaline phosphatase (red). CAM, cellulose acetate membrane; Ob, osteoblast layer.

B. Bone formed in the mouse thigh 3 weeks after xenogeneic implantation of a soluble extract of rabbit bone matrix. Three areas of bone formation are apparent, staining with Von Kossa for phosphate (black), and for alkaline phosphatase (red): A, Osteogenic site showing alkaline phosphatase activity; B, Small mineralized area; C, Trabecular bone ossicle.

C. Radiograph of mouse implanted with devitalized bone matrix showing bone formation at the implantation site (arrow). (Courtesy of Dr. O. Nilsson).

of material to intramuscular or subcutaneous sites in animals (Figure 3). Notwithstanding the difficulties, Urist et al. (1983) and Takaoka (1987) have reported the isolation and partial characterization of human BMP and mouse osteosarcoma-derived BMP, respectively, using this technique by implantation into mice. Sen et al. (1987) used bioassay in rats by recombination with inactive bone matrix from which the osteoinductive activity was removed to define a purified and partially characterized osteogenic protein.

In vitro assay is potentially more useful for any biological activity and numerous procedures have been developed over the past few years to determine osteoinductive factors (Seyedin et al. 1983, Syftestad et al. 1984, Sato and Urist 1984, Koskinen et al. 1985). These assay procedures are based on the fact that osteogenic cells form cartilage in tissue culture (Figure 4; Urist and Nogami 1970).

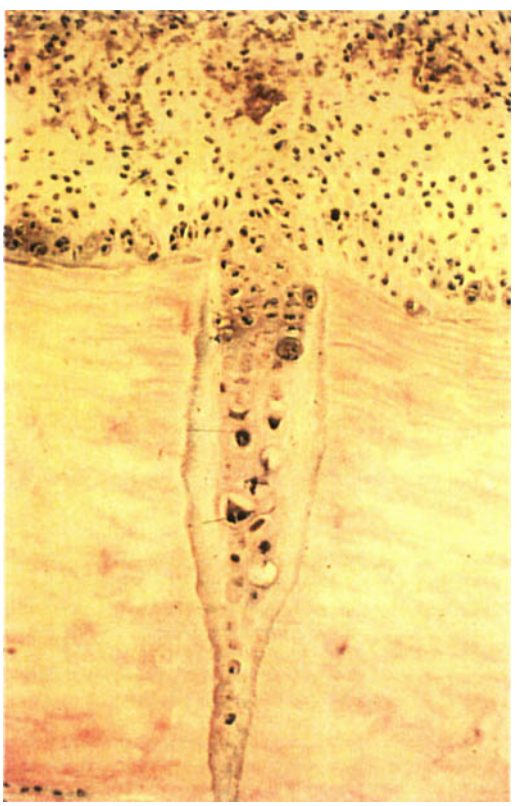

Figure 4. Cartilage formation from rat muscle fibroblasts cultured in vitro for 30 days on demineralized bone matrix. The cartilage forms from cells that are adjacent to the demineralized bone matrix and which have migrated into the less oxygenated crevice formed by a scalpel cut in this matrix. (Courtesy of Dr. O. Nilsson)

The earlier histologic criteria for cartilage induction have been superseded largely by early detection of synthesis of cartilage-specific molecules by immunochemical and molecular biological techniques. So cartilage proteoglycan and type II collagen have been the molecules detected soon after induction in vitro by fractionated bone proteins (Seyedin et al. 1983). In all of these methods, embryonic or neonatal tissues have been used for derivation of the responding cells. In one of the methods (Syftestad et al. 1984), chick-limb bud cells, which are inherently capable of differentiation into cartilage under appropriate conditions, are used. The relevance of these systems to the mechanisms occurring in vivo in adult ectopic ossification is therefore impossible to assess. What has been of great value, however, is the focusing of attention by the in vitro assays on the isolation of further factors from bone that have potentially dramatic effects on bone growth.

Recently, two cartilage-inducing factors first thought to be related to bone-inducing activity have been isolated from bone by using in vitro assays. One of them is identical to transforming growth factor ß (TGFß) and the other is a different, but homologous protein (Seyedin et al. 1986, Bentz et al. 1987). The transforming growth factors are a group of polypeptides that stimulate normal cells to grow in soft-agar culture and mimic some aspects of the transformed or malignant cell phenotype (Sporn and Todaro 1980). Their further characteristics will be noted later.

When the purified cartilage-inducing factor equivalent to TGFß was mixed with inactive bone matrix, from which all inductive activity was removed, and the mixture implanted subcutaneously into rats, bone was apparent after 14 days (Bentz et al. 1987). Incorporated into a collagen carrier, it had no inductive activity. This seems to suggest that this cartilage-inducing factor (TGFß) may potentiate residual minor activity in the extracted inactive matrix or that a cooperative effect of a number of factors is required for bone cells to be induced. It does not appear to be the single factor that initiates osteoinductive response in the genome of the potentially responsive cell.

As indicated earlier, bone matrix has been shown to contain activity that stimulates cartilage formation and growth in chick limb-bud culture in vitro. This cartilage-stimulating activity appears to be evoked by protein factors different

from those described earlier (Syftestad et al. 1987). Their chemical identities have not been described in detail, but they are likely to act at a later stage than primary induction; and some are equivalent to the bone-derived factors described later.

Local growth factors

Multifarious factors are undoubtedly involved in the complete change of cell type towards osteogenesis and in subsequent growth and development of the committed progeny. Many of these factors are probably synthesized locally, as is bone-induction activity. Recently, a host of growth factors have been detected in the matrix of bone that affect mouse fibroblast growth (Hauschka et al. 1986). Because of its mineralized nature, bone – and presumably all calcified tissue – acts as a sink for many locally produced and systemic proteins (Triffitt 1985). This also seems to be the case for growth factors. There seems to be little specificity in uptake, and a wide spectrum of activities are present. Whether growth factors are active in situ or upon bone resorption remains conjectural at this time, but growth factors must be present in the environment of cells of the osteoblast lineage. Some of the local growth factors known to affect bone-cell replication and differentiated function are described briefly below.

Bone-derived growth factors (BDGF). In vitro studies had shown at an early stage that cultured bone cells release autocrine factors that regulate bone growth (Peck and Banks 1977). These are found also in the media of cultured rat calvariae (Canalis et al. 1980). This activity has been separated into two unrelated factors, one of which is identical to TGF (Centrella and Canalis 1985). The other factor was first thought to be similar to insulin-like growth factor, or somatomedin. The latter is a family of growth hormone-dependent factors produced mainly by the liver (Phillips and Vassilopoulou-Sellin 1980).

Subsequent work has suggested this is not so, and BDGF is assumed to be different from factors found in other tissues. Strangely, however, BDGF seems to affect a variety of tissues (Canalis 1985). Although it stimulates bone cell proliferation and collagen synthesis, the action of BDGF is not specific to bone. It has also been found to stimulate synthesis of cartilage matrix components, to increase cartilage cell replication, as well as to induce mitogenic activity in normal rat kidney fibroblasts in culture. A most recent finding is that BDGF is identical to β_2-microglobulin (Canalis et al. 1987). The latter is a polypeptide bound to the major histocompatibility complex. The role of HLA antigens in disease and transplantation and their fundamental role in cell-cell recognition and immune function suggests that β_2-microglobulin may have some bearing on the genetic origin of metabolic bone disease.

Skeletal growth factor. One of the first growth factors to be isolated from human and chicken bone was named skeletal growth factor (SGF) (Farley and Baylink 1982). Its difference from BMP has been amply stated (Mohan et al. 1986, Urist et al. 1983). A specificity of SGF for skeletal tissue cells has been reported, and it does not stimulate general fibroblastic growth. The synthesis of SGF is stimulated by parathyroid hormone (Howard et al. 1980), and thus may explain the coupling between bone formation and resorption (Harris and Heaney 1969). The human factor seems to be a small molecular weight protein (m.wt. 11,000).

Growth factors derived from cartilage. A number of factors have been detected in extracts of cartilage or of chondrocytes in culture. Because bone and cartilage occur at times in intimately adjacent locations and are developmentally connected, the interactions of their respective growth factors are of considerable interest. One, named *cartilage-derived factor*, has a similar molecular weight (11,000) to SGF and resembles BDGF by its mitogenic and protein synthetic properties in culture. Other growth factors are secreted into the culture media in a range of molecular sizes (from 80–10,000 m.wt.). Their relationship to one another is unknown. Suzuki and Kato (1982) proposed the term local somatomedins to define these molecules with similar growth-promoting activities.

Platelet-derived growth factor (PDGF). This is a two-chain protein of molecular weight 30,000, and is a major mitogen secreted by platelets. It is structurally related to the product of an oncogene

encoded by simian sarcoma virus (Waterfield et al. 1983), and is a major circulating growth factor for mesenchymal cells (Stiles 1983). It is thought that proto-oncogene products are directly involved in normal cell proliferation and differentiation (Müller 1986). Certainly if the regulation of a proto-oncogene (c-fos) is altered in mice the development of bone is affected and specific lesions result (Rüther et al. 1987). PDGF is released at sites of injury during blood clotting, but its synthesis by osteoblastic bone cells other than osteosarcoma had not been shown until recently (Valentin-Opran et al. 1987). It has been shown to be present in bone matrix to the extent of 50 ng/g bone (Hauschka et al. 1986).

Transforming growth factors (TGFs). These are recently discovered molecules, and they have been referred to earlier during the discussion on bone induction. TGFß is produced by many normal and tumor cells, and the platelet is an abundant source (Derynck et al. 1985). Bone contains as much as the latter, 200 ng/g (Piez and Seyedin 1986), and it is present in and around osteoblasts and osteocytes in fetal bovine bone, as shown by immunolocalization. The synthesis of TGFß in rat bone has already been mentioned and cells growing out from human bone explants in culture (Beresford et al. 1984) also produce TGFß activity (Ibbotson et al. 1987). TGFß is released from bone cultures when resorption is stimulated, and may couple bone formation to previous resorption. When released from bone, TGFß is present as an inert complex; and acidity of the local environment, perhaps occurring during resorption, may serve to activate the factor (Pfeilschifter et al. 1987). This is of considerable interest, because acid solutions have been shown to enhance the activity of mouse tumor-derived, bone-inducing activity (Yoshikawa et al. 1986), and the cooperative activity of a number of factors may be integrated by this means. There is intense activity at the moment to learn more about the effects of TGFß on bone metabolism and how this molecule regulates proliferation and differentiation of bone cells.

Conclusions

There has thus been considerable progress in understanding the many local factors that may affect the initiation, proliferation, and differentiated functions of osteogenic cells (Figure 5). To date, the exact mechanisms of action of the local growth factors and the particular tracks in the developmental pathways that lead to bone tissue are not known. All of them mentioned are known to stimulate these processes generally, and their actions may therefore result in increased bone growth. Eventually, the exact proliferation pathway from the postulated stem cell, or the induced committed cell, to the differentiated state will be discovered. Determining the effects on this pathway of the local growth factors and of the many systemic and external factors that stimulate (Table 1) and inhibit the various stages of bone development is a monumental task for the future. It is, however, highly likely the morphogenetic factor or factors that initiate the bone-forming cell line will soon be characterized. The practical value of the inductive agent found in bone matrix is already apparent in the field of reconstructive surgery. The repair of certain skeletal defects

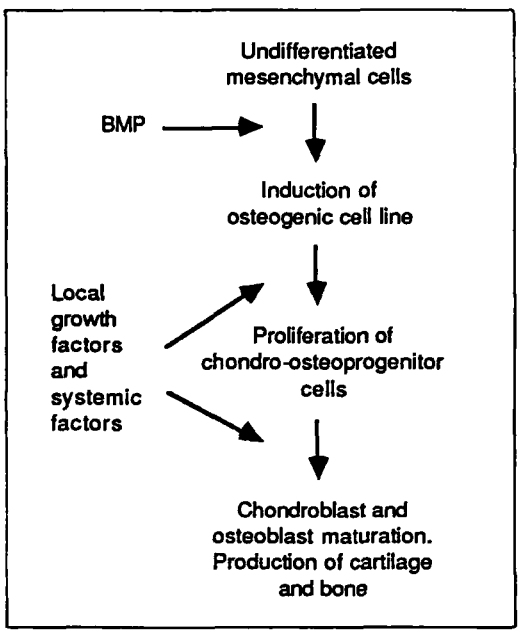

Figure 5. Stages of differentiation of bone tissue that distinguishes between a bone morphogen (BMP), which induces an undifferentiated cell to embark on a developmental pathway resulting in the progeny assuming the osteogenic cell phenotype and systemic factors that influence the proliferation and maturation of this cell line after this primary stimulation. The exact positions where these growth factors act in the cell differentiation pathways during osteogenesis are not known.

(Glowacki et al.1981), hearing defects (Frootko and Triffitt 1983, 1984), and bone defects resulting from injury, neoplasm, and infection (Urist 1976, 1986a) has been accomplished with the impure agent. The value of the purified and completely characterized product is obvious. The use of molecular biological methods of genetic engineering should eventually provide sufficient osteoinductive agent to satisfy the needs of the orthopedic surgeon, dental surgeon, basic scientist, and, not least, the patient. We should be one step nearer understanding the riddle of bone formation.

References

Allen T D. Haemopoietic microenvironments in vitro: ultrastructural aspects. Ciba Foundation Symp 1981; 84:38–67.

Amitani K, Nakata Y, Stevens J. Bone induction by lyophilized osteosarcoma in mice. Calcif Tissue Res 1974;16(4):305–13.

Anderson H C. Osteogenetic epithelial mesenchymal cell interactions. Clin Orthop 1976;(119):211–23.

Ashton B A, Allen T D, Howlett C R, Eaglesom C C, Hattori A, Owen M. Formation of bone and cartilage by marrow stromal cells in diffusion chambers in vivo. Clin Orthop 1980;(151):294–307.

Bab I, Howlett C R, Ashton B A, Owen M E. Ultrastructure of bone and cartilage formed in vivo in diffusion chambers. Clin Orthop 1984;(187):243–54.

Bauer F C, Urist M R. Human osteosarcoma derived soluble bone morphogenetic protein. Clin Orthop 1981;(154):291–5.

Bentz H, Armstrong R, Seyedin S M. Cartilage induction and differentiation: the role of bone derived cartilage inducing factor (CIF-A). In: Development and diseases of cartilage and bone matrix (Ed. Sen A, Thornhill T.) Alan R. Liss Inc. New York 1987:137–47.

Beresford J N, Gallagher J A, Poser J W, Russell R G. Production of osteocalcin by human bone cells in vitro. Effects of $1,25(OH)_2D_3$, $24,25(OH)_2D_3$, parathyroid hormone, and glucocorticoids. Metab Bone Dis Relat Res 1984;5(5):229–34.

Brånemark P I, Breine U, Johansson B, Roylance P J, Rockert H, Yoffey J M. Regeneration of bone marrow: a clinical and experimental study following removal of bone marrow by curettage. Acta Anat 1964;59:1–46.

Canalis E. Effect of growth factors on bone cell replication and differentiation. Clin Orthop 1985;(193): 246–63.

Canalis E, McCarthy T, Centrella M. Identity of bone derived growth factor with $\beta_2$microglobulin. J Bone Mineral Res 1987;2(Suppl 1):532.

Canalis E, Peck W A, Raisz L G. Stimulation of DNA and collagen synthesis by autologous growth factor in cultured fetal rat calvaria. Science 1980;210(4473): 1021–3.

Centrella M, Canalis E. Transforming and nontransforming growth factors are present in medium conditioned by fetal rat calvariae. Proc Natl Acad Sci USA 1985;82(21):7335–9.

Connors J M. Soft tissue ossification. Springer Verlag, Berlin 1983.

Derynck R, Jarrett J A, Chen E Y, Eaton D H, Bell J R, Assoian R K, Roberts A B, Sporn M B, Goeddel D V. Human transforming growth factor beta complementary DNA sequence and expression in normal and transformed cells. Nature 1985;316(6030):701–5.

Dexter T M. Stromal cell associated haemopoiesis. J Cell Physiol 1982;1(Suppl):87–94.

Farley J R, Baylink D J. Purification of a skeletal growth factor from human bone. Biochemistry 1982;21(14): 3502–07.

Friedenstein A J. Determined and inducible osteogenic precursor cells. In: Hard Tissue Growth, Repair and Remineralization. Ciba Foundation Symposium (New series) 1973;11:169–82.

Friedenstein A J. Precursor cells of mechanocytes. Int Rev Cytol 1976;47:327–59.

Friedenstein A J. Stromal mechanism of bone marrow: cloning in vitro and retransplantation in vivo. In: Immunobiology of bone marrow transplantation (Ed. Thienfelder S.) Springer Verlag, Berlin 1980:19–29.

Friedenstein A J, Piatetzky-Shapiro I I, Petrakova K V. Osteogenesis in transplants of bone marrow cells. J Embryol Exp Morphol 1986;16(3):381–90.

Frootko N J, Triffitt J T. Demineralised incus grafts in the rat middle ear. J Bone Joint Surg (Br) 1983;65:89.

Frootko N J, Triffitt J T. Osteoinduction in human demineralized malleus and incus allo-implants used to reconstruct the ossicular chain – a preliminary report. Metab Bone Dis Relat Res 1984;5:206.

Glowacki J, Kaban L B, Murray J E, Folkman J, Mulliken J B. Application of the biological principle of induced osteogenesis for craniofacial defects. Lancet 1981;1(8227):959–62.

Goldhaber P. Osteogenic induction across millipore filters in vivo. Science 1961;133:2065–7.

Ham R G, Vermett M J. Mechanisms of development. C.V. Mosby Co, St Louis 1980.

Hanamura H, Higuchi Y, Nakagawa M, Iwata H, Urist M R. Solubilization and purification of bone morphogenetic protein (BMP) from Dunn osteosarcoma. Clin Orthop 1980;(153):232–40.

Harris W H, Heaney R P. Skeletal renewal and metabolic bone disease. N Engl J Med 1969;280(5):253–9 contd.

Hauschka P V, Mavrakos A E, Iafrati M D, Doleman S E, Klagsbrun M. Growth factors in bone matrix. Isolation of multiple types by affinity chromatography on heparin Sepharose. J Biol Chem 1986;261(27): 12665–74.

Heiple K G, Herndon C H, Chase S W, Wattleworth A. Osteogenic induction by osteosarcoma and normal bone in mice. J Bone Joint Surg (Am) 1968;50(2): 311–25.

Hendry J H. Mathematical aspects of colony growth, transplantation kinetics and cell survival. In: Cell clones (Eds. Pottan C S, Hendry J H.) Churchill Livingstone, Edinburgh 1985:1–13.

Howard G A, Drivdahl R H, Baylink D J. Evidence for production and partial characterization of a coupling factor in bone metabolism. Calcif Tissue Int 1980;31:53.

Huggins C B. The formation of bone under the influence of epithelium of the urinary tract. Arch Surg 1931;22:377–408.

Huggins C B. Experimental osteogenesis. Proc Soc Exp Biol Med 1930;27:349–51.

Ibbotson K J, D Souza S M, Mundy G R. Transforming growth factors and bone. In: Development and Diseases of Cartilage and Bone Matrix (Eds. Sen A, Thornhill T.) UCLA Symposia on Molecular and Cellular Biology New Series. Alan R Liss Inc, New York 1987;46:349–64.

Koskinen K P, Kanwar Y S, Sires B, Veis A. An electron microscopic demonstration of induction of chondrogenesis in neonatal rat muscle outgrowth cells in monolayer cultures. Connect Tissue Res 1985;14(2):141–58.

Le Douarin N M. Dependence of myeloid and lymphoid organ development in stem cell seeding: investigations on mechanisms in cell marker analysis. In: Mechanisms of cell change (Eds. Ebert J, Okado T.) John Wiley & Sons, New York 1979:309–13.

Le Douarin N M, Houssaint E, Jotereau F V, Belo M. Origin of hemopoietic stem cells in embryonic bursa of Fabricius and bone marrow studied through intraspecific chimeras. Proc Natl Acad Sci USA 1975;72(7):2701–5.

Luria E A, Panasyuk A F, Friedenstein A J. Fibroblast colony formation from monolayer cultures of blood cells. Transfusion 1971;11(6):345–9.

Maloney M A, Patt H M. Bone marrow restoration after localized depletion. Cell Tissue Kinet 1969;2:29–38.

Maximow A. Cultures of blood leucocytes; from lymphocyte and monocyte to connective tissue. Arch Exp Zellforsch 1927–1928;5:169–268.

Mohan S, Jennings J C, Linkhart T A, Baylink D J. Isolation and purification of a low molecular weight skeletal growth factor from human bones. Biochim Biophys Acta 1986;884(2):234–42.

Müller R. Protooncogenes and differentiation. Trends Biochem Sci 1986;11:129–32.

Owen M. Histogenesis of bone cells. Calcif Tissue Res 1978;25(3):205–7.

Owen M. The origin of bone cells in the postnatal organism. Arthritis Rheum 1980;23(10):1073–80.

Owen M. Lineage of osteogenic cells and their relationship to the stromal system. In: Bone and Mineral Research (Ed. Peck W A.) Elsevier 1985;3:1–25.

Patt H M, Maloney M A. Bone marrow regeneration after local injury: a review. Exp Hematol 1975;3(2):135–48.

Patt H M, Maloney M A. Evolution of marrow regeneration as revealed by transplantation studies. Exp Cell Res 1972;71(2):307–12.

Peck W A, Banks J K. Proliferation and specialization of bone cells cultured in serum free medium. In: Mechanisms of Localized Bone Loss, Calcified Tissue Abstracts (Eds. Horton J, Tarpley T, Davis W.) 1977:3–12.

Pfeilschifter J, Bonewald L, Mundy G R. TGF is released from bone with one or more binding proteins which regulate its activity. J Bone Mineral Res 1987;2(Suppl 1):532.

Phillips L S, Vassilopoulou-Sellin R. Somatomedins (Part 1). N Engl J Med 1980;302(7):371–80.

Phillips L S, Vassilopoulou Sellin R. Somatomedins (Part 2). N Engl J Med 1980;302(8):438–46.

Piersma A H, Brockbank K G, Ploemacher R E, van Vliet E, Brakel van Peer K M, Visser P J. Characterization of fibroblastic stromal cells from murine bone marrow. Exp Hematol 1985;13(4):237–43.

Piersma A H, Ploemacher R E, Brockbank K G, Nikkels P G, Ottenheim C P. Migration of fibroblastoid stromal cells in murine blood. Cell Tissue Kinet 1985;18(6):589–95.

Piez K A, Seyedin S M. Osteoinduction and cartilage inducing factors from bone. Am Soc Bone Mineral Research Proc 8th Annual Meet, Sandoz Workshop on Growth Factors and Bone Formation, Anaheim, Cal. 1986:17–20.

Rüther U, Garber C, Komitowski D, Müller R, Wagner E F. Deregulated c-fos expression interferes with normal bone development in transgenic mice. Nature 1987;325(6103):412–6.

Sahebekhtiari H A, Tavassoli M. Studies on bone marrow histogenesis: morphometric and autoradiographic studies of regeneration marrow stroma in extramedullary autoimplants and after evacuation of marrow cavity. Cell Tissue Res 1978;192(3):437–50.

Sampath T K, Reddi A H. Dissociative extraction and reconstitution of extracellular matrix components involved in local bone differentiation. Proc Natl Acad Sci USA 1981;78(12):7599–603.

Sato K, Urist M R. Bone morphogenetic protein induced cartilage development in tissue culture. Clin Orthop 1984;(183):180–7.

Sen A, Walker M A, Einarson B. Purification and partial characterization of a novel osteogenic protein. In: Development and Diseases of Cartilage and Bone Matrix, UCLA. Symposia on Molecular and Cellular Biology New Series. Alan R Liss Inc. New York 1987;46:201–20.

Seyedin S M, Thompson A Y, Rosen D M, Piez K A. In vitro induction of cartilage-specific macromolecules by a bone extract. J Cell Biol 1983;97(6):1950–3.

Seyedin S M, Thompson A Y, Bentz H, Rosen D M, McPherson J M, Conti A, Siegel N R, Galluppi G R, Piez K A. Cartilage-inducing factor A. Apparent identity to transforming growth factor beta. J Biol Chem 1986;261(13):5693–5.

Slack J M. Embryology: we have a morphogen! (news). Nature 1987;327(6123):553–4.

Smith R, Triffitt J T. Bones in muscles: the problems of soft tissue ossification. Q J Med 1986;61:985–90.

Sporn M B, Todaro G J. Autocrine secretion and malignant transformation of cells. N Engl J Med 1980;303(15):878–80.

Stiles C D. The molecular biology of platelet derived growth factor. Cell 1983;33(3):653–5.

Stoker M. Growth factors in biology and medicine. Ciba Found Symp 1985;116:1–2.

Suzuki F, Kato Y. Action of growth factors on cartilage: Discovery of local somatomedins in fetal bovine cartilage. In: Int Cong Ser No. 598 Endocrinology (Eds. Shizume K, Imura H, Shimizu N.) Excerpta Medica, Amsterdam 1982:239–43.

Syftestad G T, Triffitt J T, Urist M R, Caplan A I. An osteo-inductive bone matrix extract stimulates the in vitro conversion of mesenchyme into chondrocytes. Calcif Tissue Int 1984;36(5):625–7.

Syftestad G T, Lucas P A, Ohgushi H, Caplan A I. Chondrogenesis as an in vitro response to bioactive factors extracted from adult bone and non skeletal tissues. In: Development and Diseases of Cartilage and Bone Matrix (Eds. Sen A, Thornhill T.) UCLA Symposia on Molecular and Cellular Biology New Series. Alan R Liss Inc. New York1987;46:187–99.

Takaoka K. Personal communication 1987.

Tavassoli M, Crosby W H. Transplantation of marrow to extramedullary sites. Science 1968;161(836):54–6.

Thaller C, Eichele G. Identification and spatial distribution of retinoids in the developing chick limb bud. Nature 1987;327(6123):625–8.

Triffitt J T. Receptor molecules, coprecipitation and ion exchange processes in the deposition of metal ions in bone. In: Metals in Bone (Ed. Priest M.) MTP Press Ltd, Lancaster 1985:3–20.

Trueta J. Studies on the development and decay of the human frame. Heinemann, London 1968:7.

Urist M R. Bone: formation by autoinduction. Science 1965;150(698):893–9.

Urist M R, Hay P H, Dubuc F, Buring K. Osteogenetic competence. Clin Orthop 1969;64:194–220.

Urist M R. Practical applications of basic research on bone graft physiology. AAOS Instructional Course Lectures 1976;25:1–26.

Urist M R, Hudak R T. Radioimmunoassay of bone morphogenetic protein in serum: a tissue specific parameter of bone metabolism. Proc Soc Exp Biol Med 1984;176(4):472–5.

Urist M R, Mikulski A J. A soluble bone morphogenetic protein extracted from bone matrix with a mixed aqueous and nonaqueous solvent. Proc Soc Exp Biol Med 1979;162(1):48–53.

Urist M R, Nogami H. Morphogenetic substratum for differentiation of cartilage in tissue culture. Nature 1970;225(237):1051–2.

Urist M R, DeLange R J, Finerman G A. Bone cell differentiation and growth factors. Science 1983;220(4598):680–6.

Urist M R, Kovacs S, Yates K A. Regeneration of an enchondroma defect under the influence of an implant of human bone morphogenetic protein. J Hand Surg (Am) 1986;11(3):417–9.

Urist M R, Hudak R T, Huo Y K, Rasmussen J K. Osteoporosis: a bone morphogenetic protein autoimmune disorder. In: Normal and Abnormal Bone Growth: bone and clinical research Alan R Liss Inc., New York 1985:77–96.

Urist M R, Chang J J, Huo Y K, Brownell A G, DeLange R J. Methods of preparation and bioassay of bone morphogenetic protein and polypeptide fragments. Methods Enzymol 1986. In press.

Urist M R, Conover M A, Lietze A, Triffitt J T, DeLange R. Partial purification and characterization of bone morphogenetic protein. In: Hormonal Control of Calcium Metabolism (Eds. Cohn D, Talmage R, Matthews J L.) Excerpta Medica, Amsterdam 1981:307–14.

Urist M R, Huo Y K, Brownell A G, Hohl W M, Buyske J, Lietze A, Tempst P, Hunkapiller M, DeLange R J. Purification of bovine bone morphogenetic protein by hydroxyapatite chromatography. Proc Natl Acad Sci USA 1984;81(2):371–5.

Urist M R, Lietze A, Mizutani H, Takagi K, Triffitt J T, Amstutz J, DeLange R, Termine J, Finerman G A. A bovine low molecular weight bone morphogenetic protein (BMP) fraction. Clin Orthop 1982;(162):219–32.

Urist M R, Sato K, Brownell A G, Malinin T I, Lietze A, Huo Y K, Prolo D J, Oklund S, Finerman G A,

DeLange R J. Human bone morphogenetic protein (hBMP). Proc Soc Exp Biol Med 1983;173(2):194–9.

Valentin-Opran A, Delgado R, Valente T, Mundy G R, Graves D T. Autocrine production of platelet derived growth factors (PDGF) like peptides by cultured normal human bone cells. J Bone Mineral Res 1987;2(Suppl 1):533.

Waterfield M D, Scrace G T, Whittle N, Stroobant P, Johnsson A, Wasteson A, Westermark B, Heldin C H, Huang J S, Deuel T F. Platelet-derived growth factor is structurally related to the putative transforming protein p28sis of simian sarcoma virus. Nature 1983;304(5921):35–9.

Weiss L. Haemopoiesis in mammalian bone marrow. Ciba Found Symp 1981;84:5–21.

Wilson D. The origin of the endothelium in the developing marginal vein of the chick wing bud. Cell Differ 1983;13(1):63–7.

Wlodarski K. The inductive properties of epithelial established cell lines. Exp Cell Res 1969;57(2):446–8.

Yoshikawa H, Takaoka K, Shimizu N, Ono K. Acid solutions enhance bone inducing activity of a murine osteosarcoma. Bone 1986;7(2):125–8.