Chapter 22

MATRIX-INDUCED ENDOCHONDRAL BONE DIFFERENTIATION: A MODEL FOR REGENERATIVE GROWTH CONTROL BY EXTRACELLULAR MATRIX

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Introduction

Among the various tissues in the vertebrate body, the skeletal system is endowed with the most potential for regeneration and repair. Although certain tissues, such as liver, are capable of regeneration, the extent of this process is truly spectacular in the limb of tailed amphibians. In the higher vertebrates, despite the lack of complete regeneration of limbs, the skeleton has retained the potential for regenerative growth as exemplified by fracture healing. Regeneration is a fascinating biological phenomenon and has attracted its share of devotees (Morgan, 1901; Hay, 1966; Schmidt, 1968; Goss, 1969; Rose, 1970; Polezhaev, 1972; Singer, 1973; Thornton and Bramley, 1973; Mattson, 1976) who have provided detailed descriptions of the process. Despite numerous studies, the basic mechanisms underlying regeneration are still not understood. What is the initial signal for blastema formation: What mechanisms are operative in the initial migration of cells and their subsequent proliferation? What mechanisms might be involved in the local growth control during regeneration? These are just a few questions to set the stage for the present discourse.

The aim of this chapter is to describe experiments in our laboratory on matrix-induced endochondral bone differentiation and point out the relevance of cell-free extracellular matrix to regenerative growth control. Subcutaneous implantation of demineralized bone matrix results in the induction of a sequential morphogenetic cascade culminating in the formation of an ossicle of endochondral bone replete with bone marrow (Reddi and Huggins, 1972a, 1975; Reddi and Anderson, 1976; Reddi, 1979). The differentiative sequence of this inductive phenomenon is similar to those observed during amphibian limb regeneration and fracture healing in man. The experimental advantages of the matrix-induced bone forming system follow: (1) it permits sequential observations of early regenerative phases of bone in an extraskeletal locale; (2) it affords a method to experimentally dissect the various temporal phases of a compli-

cated biological process; and (3) it allows a metabolic approach to the study of discrete stages of the problem with combined chemical and morphological techniques. The accrued results have immense implications for regeneration, growth control, fracture healing, bone grafts, dental implants, and a host of other biomedical problems. We will present the concept that the cell-free extracellular collagenous matrix obtained from demineralized diaphyseal bone specifies local positional information for regeneration and fracture healing. Similar mechanisms may be operative during normal morphogenetic phases of embryonic development (Reddi, 1976).

Matrix-Induced Bone Differentiation

Experimental Methods

Huggins et al. (1936) were the first to describe new bone formation after autologous intramuscular transplantation of canine incisor teeth. Urist (1965) discovered the osteogenic potential of demineralized, lyophilized segments of teeth and bone. We have developed techniques for rapid induction of cartilage, bone, and bone marrow by subcutaneous implantation of demineralized, diaphyseal bone matrix of discrete particle size into allogeneic rats (Reddi and Huggins, 1972a).

Diaphyseal shafts from long bones of rats were cleaned free of bone marrow and periosteum by repeated washing in distilled water and dehydrated in ethanol and anhydrous ether. Pulverization of the bone in a micromill yielded powders that were sieved to a discrete size that ranged from 74 to 420 μm. The powders were demineralized in 0.5*M* HCl or 0.5*M* EDTA, pH 7.4, resulting in samples of similar biological potency (Reddi, 1974). However, the former method is rapid (2 hours at 25°C) and inexpensive. Following demineralization, the matrix was washed in water and dehydrated in ethanol and ether (Reddi and Huggins, 1972a). Demineralized bone matrix of this sort is devoid of detectable calcium and inorganic phosphate. The amino acid composition revealed the matrix to be essentially collagenous (Reddi, 1976). The total anthrone-reactive carbohydrates ranged between 0.7 to 1.0 percent by weight and consisted of glucose, galactose, and mannose.

Subcutaneous implantation of demineralized diaphyseal bone matrix into young allogeneic recipients resulted in new endochondral bone formation locally (Reddi and Huggins, 1972a; Reddi and Anderson, 1976). Our general experimental approach has been to characterize the matrix-induced bone development in precise cellular phenotypes and biochemical terms. During the course of delineating the step-wise changes in the developmental sequence, we have probed the underlying mechanisms. The long-term goal of our laboratory is to understand the cell

biology of bone development and to explore the molecular mechanisms of induction of bone differentiation.

Sequential Cellular Changes

On subcutaneous implantation of collagenous diaphyseal bone matrix, there was an almost instantaneous formation of a blood clot. On day 1, a buttonlike planoconvex plaque (implant) was observed. It consisted of implanted matrix and numerous polymorphonuclear leukocytes enmeshed in an irregular fibrin network (Reddi and Anderson, 1976). On days 3 and 4 spindle-shaped mesenchymal cells were in the vicinity of the implanted matrix, interacted with the matrix, and subsequently proliferated. The first chondroblasts were evident on day 5 and chondrocytes were abundant on days 7 and 8 in the intermatrix area. On day 9, extensive calcification of the newly formed cartilagenous matrix was commonly observed, and vascular invasion into cartilage occurred. With the appearance of invading capillaries, chondrolytic foci were seen. In adjacent areas in close proximity to the capillary endothelium and pericytes, basophilic osteogenic cells and osteoblasts were seen; these cells were more numerous on days 10 and 11. New bone formation proceeded by appositional growth on calcified cartilage spicules and on the surface of implanted bone matrix. Concurrently, on days 12-18, bone remodeling occurred with formation of sinusoids that enlarged by confluence, presenting a "swiss-cheese"-like appearance. Extravascular colonies of hematopoiesis were observed and consisted of basophilic hemocytoblasts and developing erythroid and granulocytic cells (Reddi and Huggins, 1975; Reddi and Anderson, 1976).

The ability to induce de novo bone formation is also shared by tooth matrix. The osteogenic potency is approximately equal in both bone and tooth matrix. Mineralized matrix is incapable of bond induction. The mineral phase has to be removed for the expression of osteogenic potential (Reddi and Huggins, 1972b; Reddi, 1974, 1975). Among the different types of bone in the mammalian body, compact bone is most active, followed by cancellous bone from scapula and pelvis and membranous bone from the calvarium (Reddi, 1975a,b, 1976). Matrices from tail tendon, Achilles tendon, cartilage, skin, and aorta are devoid of osteogenic properties (Reddi, 1975a).

Biochemical Changes

Alkaline Phosphatase and Mineralization: A several-fold increase in alkaline phosphatase activity was observed prior to bone mineralization (Reddi and Huggins, 1972a). This increase was followed by incorporation of ^{32}P and ^{45}Ca into bone mineral fraction (Reddi and Huggins, 1972a; Reddi, 1976) (Fig. 22-1). Bone formation was also correlated with a de-

crease in lactate dehydrogenase activity (Reddi and Huggins, 1971) and an increase in citric acid levels (Reddi and Huggins, 1972b). Although the precise role of alkaline phosphatase in mineralization is not known, it is likely that it plays a role in hydrolyzing pyrophosphate and reducing the inhibitory effect of this compound. It could also function as a phosphotransferase system.

Collagens: collagens and proteoglycans are the two major classes of extracellular matrix macromolecules in skeletal tissues. The occurrence and distribution of several genetically distinct collagen types is now well established (Miller, 1976). An analysis of changes in collagen types during bone morphogenesis may lead to a better understanding of the role of these macromolecules in development. The localization of types I, II, III, and IV collagens during matrix-induced cartilage, bone, and bone marrow formation was studied by specific immunofluorescence (Fig. 22-1). On day 3, type III collagen was detected (Reddi et al., 1977). Type II collagen was detected in the cartilage matrix with the onset of chondrogenesis. Vascular invasion of the implant on day 9 was monitored by localization of basement

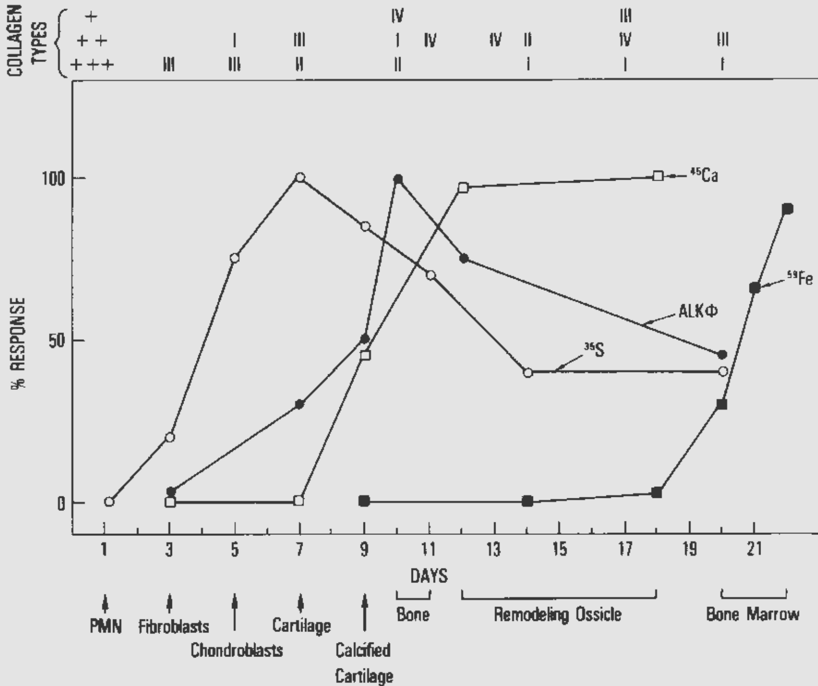

Figure 22-1. Changes in $^{35}SO_4$ incorporation into proteoglycans and ^{45}Ca incorporation into bone mineral plotted from our previous data (Reddi and Huggins, 1972a; Reddi, 1976). The ^{59}Fe incorporation into heme as an index of erythropoiesis was taken from Reddi and Anderson (1976). These quantitative indicators were correlated with the phenotype changes.

membrane type IV collagen (Foidart and Reddi, 1980). Type I collagen was demonstrated in the newly deposited bone matrix coating the surfaces of cartilage spicules on days 10-11. Type III collagen was localized as a fibrous array in hematopoietic bone marrow.

PROTEOGLYCANS: Prompted by the recent advances in cartilage proteoglycan biochemistry (Hascall and Heinegard, 1978), we have examined the biosynthesis of proteoglycans labeled *in vivo* with $^{35}SO_4$ and then by associative and dissociative extraction techniques and chromatography (Reddi et al., 1978) during matrix-induced endochondral bone development. During maximal chondrogenesis on day 7 (Fig. 21-1), the elution profile of $^{35}SO_4$-labeled proteoglycans on Sepharose® 2B was identical to the proteoglycans isolated from rat femoral head cartilage. On day 9, the hypertrophic cartilage matrix underwent calcification and at this time there was a decline in synthesis of cartilage-type proteoglycans and an increase in smaller size proteoglycans. The formation of these smaller bone-type proteoglycans increased further on days 11 to 14. The component chondroitin sulfate chains had an average molecular weight of 50,000 compared to 25,000 for chondroitin-sulfate chains of cartilage proteoglycans. Autoradiography of $^{35}SO_4$-labeled implants revealed that osteoblasts were the site of synthesis of bone-type proteoglycans. More recently the cartilage-specific proteoglycans and link protein were localized by specific immunofluorescence methods (Reddi and Poole, unpublished observations). There was no cross reaction with bone-type proteoglycans in developing bone.

FIBRONECTIN: Fibronectin is a pericellular glycoprotein that is also present in a circulating form in the blood plasma and was previously known as cold insoluble globulin (Yamada and Olden, 1978; Vaheri et al., 1978; Kleinmann et al., 1979; Pearlstein et al., 1980). It has been shown to function *in vitro* as an adhesive protein for cell-substratum or cell-cell interactions. Collagen is the usual extracellular substratum for cells and has an affinity for fibronectin. In view of the collagen-fibronectin interaction, we examined the synthesis and function of fibronectin during collagenous bone matrix-induced cartilage and bone development by labeling with [^{35}S]-methionine. The subcutaneously implanted demineralized bone-matrix bound circulating fibronection, which may be an important initial requirement for cell attachment to the matrix (Wiss and Reddi, 1980a). Other studies have demonstrated that fibronectin functions as α-opsonic protein during phagocytosis and it is likely that this function is critical for wound debridement (Sabe et al., 1978). Recently fibronectin has been shown to be a chemotactic macromolecule (V. Gauss-Muller et al., unpublished observations), and therefore, it may be involved in early phases of mesenchymal cell recruitment during wound healing, regeneration, and fracture healing. Fibronectin was also localized by specific im-

munofluorescence in the extracellular matrix of proliferating mesenchymal cells (Weiss and Reddi, 1980a). The temporal appearance of fibronectin on day 3 coincides with the appearance of large amounts of type III collagen (Steinmann and Reddi, 1980). It is noteworthy in this connection that fibronectin binds most avidly to native type III collagen compared to native type I and II (Ruoslahti and Engvall, 1978 and Hormann and Jilek, 1978).

NON-COLLAGENOUS PROTEINS: (1) γ-carboxyglutamic acid (Gla) is a constituent of the noncollagenous bone protein osteocalcin (Hauschka et al., 1975; Price et al., 1976). The appearance of γ-carboxyglutamic acid during matrix-induced endochondral bone development was examined (Hauschka and Reddi, 1980). The residual Gla in the demineralized bone matrix was lost rapidly upon implantation. Gla levels were basal during chondrogenesis and begin to increase on day 9 and thereafter during mineralization of bone.

(2) Laminin is a noncollagenous glycoprotein component of a basement membrane producing murine tumor (Timpl et al., 1979). It consists of at least two polypeptide chains (220 kd and 440 kd) linked by disulfide bonds and has been localized in the lamina rara of the basement membrane. Laminin was localized by specific immunofluorescence to the invading blood vessels and was dependent on the presence of pituitary growth hormone (Foidart and Reddi, 1980). The endothelial cell proliferation and invasion was further correlated with localization of blood coagulation factor VIII (Foidart and Reddi, 1980).

Hormonal Control

Matrix-induced bone formation, like regeneration of newt limb (Tassava, 1969, is pituitary growth hormone dependent (Reddi, 1974; Reddi and Sullivan, 1980). There is an additive effect of growth hormone and thyroid stimulating hormone (TSH) (Reddi and Sullivan, 1980). Whereas growth hormone and TSH were adequate to completely restore the ^{45}Ca incorporation in tibial metaphysis in hypophysectomized rats, it was inadequate to support the initiation of mineralization in developing implants. These results imply that additional pituitary factors govern initial bone formation and mineralization (Reddi and Sullivan, 1980).

The local influence of corticosteroids such as dexamethasone on bone formation was investigated using various biochemical parameters at discrete stages of development. Ornithine decarboxylase (ODC) activity is a convenient indicator of mesenchymal cell proliferation prior to chondrogenesis and osteogenesis (Rath and Reddi, 1978). Locally administered dexamethasone inhibited mesenchymal cell proliferation on days 3-4 as indicated by ODC activity (Rath and Reddi, 1979a), and chondrogenesis was subsequently impaired. Administration of dexamethasone on days 7

through 10 impaired osteogenesis by inhibition of mesenchymal cell proliferation on day 8. However, if dexamethasone is administered after mesenchymal cell proliferation on days 10 and 11, osteogenesis was not affected, indicating that the action of dexamethasone on endochondral bone is primarily mediated by inhibition of mesenchymal cell proliferation prior to osteogenesis (Rath and Reddi, 1979a).

It is well known that the incidence of osteoporosis in diabetics is higher than in age- and sex-matched controls. However, the underlying mechanisms have not been examined. The influence of streptozotocin-induced diabetes on matrix-induced bone formation was studied (Weiss and Reddi, 1980b). The matrix-induced endochondral bone formation was decreased due to inhibition of insulin-dependent mesenchymal cell proliferation and the consequent decrease in chondrogenesis and osteogenesis (Weiss and Reddi, 1980b). Local injection of insulin at the implant site at doses without any effect on plasma glucose corrected the impaired bone mineralization indicating the critical importance of insulin (Weiss and Reddi, unpublished observations).

Nutrititional Influence

It is well known that vitamins and minerals are critical in the regulation of bone mineralization. Investigation of the influence of various nutritional deficiencies requires an experimental model in which *de novo* mineralization of bone can be manipulated by nutritional factors. The initiation of the mineralization occurs in the mammalian fetus *in utero*. In order to avoid the technical difficulties of performing nutritional experiments with prenatal fetuses, we have examined the influence of various nutritional states on matrix-induced bone development systems.

The influence of magnesium deficient diet (50 ppm/Mg) for seven days on matrix-induced bone development compared to control diet (1000 ppm Mg) was investigated. Magnesium deficient diet markedly retarded the histologically observed bone mineralization and ^{45}Ca incorporation (Schwartz and Reddi, 1979). There was a virtual absence of bone marrow in day 20 plaques in magnesium-deficient rats. In other studies, fluoride feeding (50 ppm) in drinking water delayed the onset of mineralization in matrix-induced bone (Eanes and Reddi, 1979). In other experiments, the role of inorganic phosphate for mineralization of cartilage was investigated, and it was found that hypophosphatemia severely impaired bone mineralization (Reddi and Binderman, 1979).

Influence of Age

The rate of regeneration of limbs decreases with age in amphibians and in growing pouch opossum (Mizell, 1968). In view of this we compared the osteogenic potency of bone matrix in young (28-35 day old) and old (>750

days) rats. We observed that the chondrogenesis and osteogenesis were delayed and the response markedly diminished in old rats. In the converse experiment, bone matrix from two year old rat was osteoinductively weaker than an adult (6 month) rat. These observations will permit us to explore the basis for loss of osteogenic potency and attempts to restore it by hormonal and nutritional manipulation.

Mechanism of Action of Matrix

The precise molecular mechanism of the action of matrix on cells to trigger the sequential cell differentiation is now known. However, our knowledge of this matrix-cell interaction is continually evolving and will be summarized in this section together with some current experimental approaches. The surface charge on the matrix is crucial as alteration by the anionic azo dye, Evans blue (Reddi and Huggins, 1974), or heparin (Reddi, 1975) is inhibitory to induction of bone. The physical dimensions of the matrix is critical and it is plausible that anchorage-dependent phenomena (Stoker et al., 1968) regulate the kinetics and yield of the induced bone (Reddi, 1976). Recent experiments have revealed that there is a matrix size-dependent increase in cell proliferation (Reddi, 1980 unpublished observations). The glycoproteins associated with matrix are necessary for bone induction as oxidation by periodate or galactose oxidase abolishes the osteoinductive properties of the matrix (Reddi, 1976). In this connection it is instructive to ask the question: Is collagenous matrix necessary? This is especially relevant in view of the reported isolation of bone morphogenetic protein (BMP) by Urist and coworkers (Urist et al., 1973, 1979). In these experiments the bone matrix was solubilized by collagenous digestion, partially purified by chromatography implanted in double-walled diffusion chambers, and assayed by histological examination. It should be pointed out that the bioassayed fractions were really composed of a mixture of proteins that has bone morphogenetic activity. Further, in the absence of an objective quantitative technique for measuring bone yield, it is difficult to assess the increase (or decrease) in specific activity of solubilized preparations from that of the original matrix.

The current working hypothesis of our laboratory is that collagen is necessary for cell attachment (Klebe, 1974; Reddi, 1976; Kleinmann et al., 1979) and associated glycoproteins are involved in the induction of cell differentiation. Further, in the context of the experiments concerning anchorage-dependent differentiation (Reddi, 1976), we believe collagenous extracellular matrix provides the framework to which cells attach and interact as a prelude to more specific information transfer that results in cell proliferation and altered gene expression in the responding fibroblasts. In view of the local nature of regenerative growth and fracture healing, it is likely that collagens insolubilize and restrict the mobility of

morphogenetically crucial macromolecules that may specify positional information (Wolpert, 1969).

The major phases of the matrix-induced bone development are (1) chemotaxis and cell migration, (2) cell proliferation, and (3) cell differentiation. Previous studies have stressed the chemotactic property of the matrix *in vivo* (Reddi and Huggins, 1972a). A partial solubilization of matrix components was obtained by 4.0*M* guanidine hydrochloride (Anastassiades et al., 1978) and the chemotactic potency of the macromolecular fraction was examined. It was found to be chemotactic for rat embryo fibroblasts (Reddi and Seppa, 1980, unpublished observations).

Radioautographic and biochemical studies have demonstrated that collagenous bone matrix is a local mitogen (Rath and Reddi, 1979b). These results revealed the local influence of bone matrix on mesenchymal cell proliferation and imply a role for this extracellular matrix in anchorage-dependent events in cell growth and differentiatiou. The importance of collagenous matrix in development and in the specification of positional information (Wolpert, 1969) has been discussed before (Reddi, 1976).

Bone matrix-induced endochondral bone formation has immense clinical implications and may have future clinical applications. It is known that bone shavings heal skull defects in rats and dogs (Polezhaev, 1972). It is reasonable to contemplate the possible applications in non-union of fractures, periodontal lesions, bone augmentation in acquired and congenital craniofacial defects, the realm of plastic and reconstructive surgery, and a host of metabolic bone disorders. Apart from its potential clinical uses, the matrix-induced sequential cell differentiation is a useful paradigm for a better understanding of matrix-cell interactions *in vivo* and the role of extracellular matrix in growth control and morphogenesis. In conclusion, although many of the questions posed in the introduction are still unanswered, future work with matrix-induced bone differentiation with new techniques is hound to be exciting, informative, and useful.

Summary

Skeletal tissues have the potential for regenerative growth and repair. Despite numerous studies, the basic mechanisms underlying regeneration are still not understood. We have investigated several aspects of endochondral bone differentiation by subcutaneous implantation of demineralized bone matrix in rats. The sequential morphogenetic changes induced by the bone matrix are similar to those observed during limb regeneration in amphibians and fracture-healing in man.

Subcutaneous implantation of demineralized powder of rat diaphyseal bone matrix induces endochondral bone differentiation on an invariant temporal sequence. On day 1 there is a transient appearance of polymorphonuclear leukocytes in the implant. Mesenchymal cells appear in close

contiguity with the matrix on day 3, proliferate, and then emerge as chondroblasts on days 5 and 6 and differentiate and elaborate cartilage matrix on day 7. On day 9 the hypertrophied cartilage matrix undergoes mineralization. Concomitant with vascular invasion, bone-forming osteoblasts appear on day 10. The newly formed bone is remodeled by osteoclasts (days 12-18) and there is a concurrent dissolution of the implanted matrix. By day 21 hematopoietic bone marrow differentiation occurs in the ossicle.

The histochemical and biochemical changes during such matrix-induced bone differentiation were studied. The mechanism of action of the matrix on mesenchymal cells is not known. However, the surface charge characteristics and physical size are critical for bone induction. The collagenous bone matrix is a local mitogen as demonstrated by (^{3}H)-thymidine incorporation and ornithine decarboxylase activity. These results imply that positional information for regeneration and fracture-healing is specified by the extracellular collagenous matrix.

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