Journal of Cellular Biochemistry 55:350–357 (1994)

Role of Active and Latent Transforming Growth Factor β in Bone Formation

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Abstract At first reading the statement "TGFβ stimulates bone formation but inhibits mineralization" may appear to be an oxymoron. However, the bone formation process can take weeks to months to complete, and the unique properties of TGFβ allow this factor to be stored in bone matrix in a latent form, ready to be activated and inactivated at key, pivotal stages in this long process. TGFβ may act to trigger the cascade of events that ultimately leads to new bone formation. However, once this process is initiated, TGFβ must then be inactivated or removed because if present in the later stages of bone formation, mineralization will be inhibited. The unique properties of TGFβ and its role in bone remodeling are the subject of this review.

Key words: TGFβ, bone formation, mineralization, osteoblasts, osteoclasts

PHYSICAL PROPERTIES OF TGFβ

The transforming growth factor β (TGFβ) gene family consists of four distinct proteins, TGFβ1, -2, -3, and -5. Mammalian TGFβ1 and chicken TGFβ4 are products of homologous rather than duplicated genes [Burt and Paton, 1992]. TGFβ1, -2, and -3 are differentially expressed in mammalian tissues [Miller et al., 1989], and each binds with different affinities to the TGFβ receptors [López-Casillas et al., 1993] and each have slightly different biologic effects [Graycar et al., 1989]. Greater homology exists between the mature regions as compared to the precursor regions, suggesting that the precursor regions may provide distinct functions.

Active TGFβ is a 25 kD homodimer which contains nine cysteins must be dissociated from a secreted latent complex to become biologically active. The precursor or latency associated peptide is all that is necessary to confer latency to TGFβ, and a 100 kD precursor form of recombinant latent TGFβ is produced by transfected Chinese hamster ovary cells [Gentry et al., 1987] (see Fig. 1). In most cell types, however, the latent TGFβ complex also contains a protein called the TGFβ binding protein. Fibroblasts produce a latent complex containing a 190 kD binding protein which is covalently attached to one of the precursor proteins [Miyazono et al., 1988; Kanzaki et al., 1990; Tsuji et al., 1990], whereas platelets produce a latent complex containing a 130 kD truncated form of the binding protein [Wakefield et al., 1988; Kanzaki et al., 1990]. Bone cells produce the 100 kD precursor latent complex similar to recombinant latent TGFβ [Bonewald et al., 1991] and also make the fibroblast but not the platelet form of latent TGFβ [Dallas et al., 1994].

Bone cells are unique in making two latent forms of TGFβ, one which contains TGFβ binding protein and one which does not. The function of the latent TGFβ binding protein is unknown. It does not confer latency to the complex, but clues to its function in bone are being obtained from immunohistochemical studies of mineralizing fetal rat calvarial bone cells. Our preliminary data show that this binding protein forms long fibrillar strands in these cultures which span between nodules and that these fibrillar strands may play a role in initiating and directing new nodule formation [Dallas et al., 1993].

ACTIVATION OF LATENT TGFβ

Such an abundant growth factor with such potent effects on cells must be tightly regulated. This regulation is achieved through latency.

Received February 17, 1994; accepted February 22, 1994.

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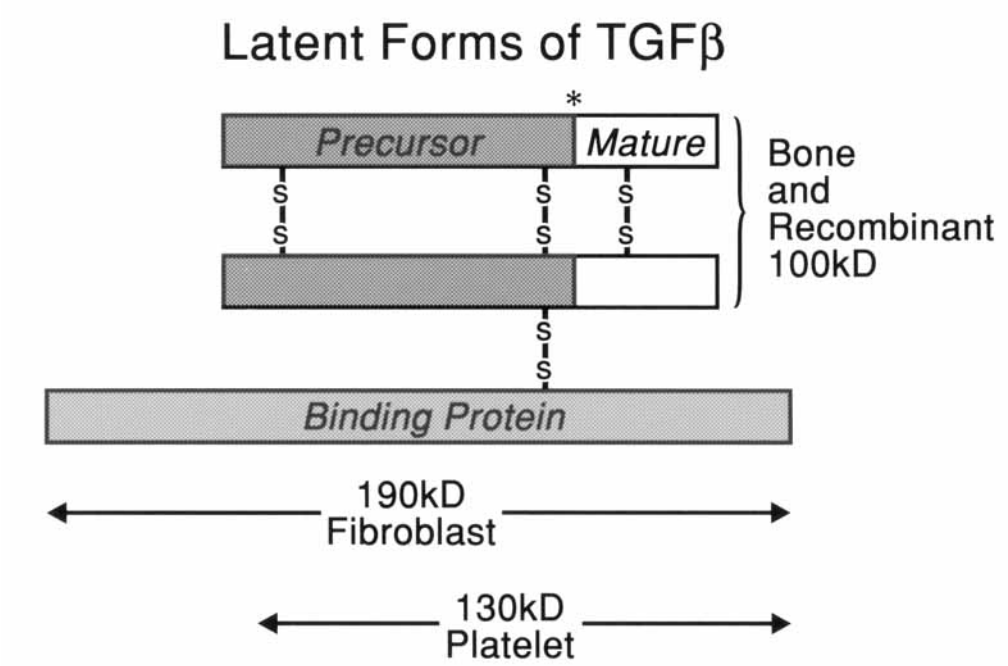

Fig. 1. A schematic representation of the various forms of latent TGFβ produced by different cell types. Bone cells produce the 100 kD latent complex lacking the latent TGFβ binding protein and the 290 kD latent complex containing the 190 kD nontruncated form of the binding protein. *, internal cleavage site between precursor (also known as latency associated peptide) and mature TGFβ which is cleaved before the complex is secreted by the cell. The mature TGFβ homodimer must be dissociated from the precursor to become biologically active. The latent TGFβ binding protein does not confer latency to the complex and is not necessary for processing and secretion in bone cells. Preliminary studies in our lab suggest a role in organizing matrix into a state where bone formation and mineralization can occur.

There are many ways in which cells or tissues "control" TGFβ. TGFβ is generally secreted and stored by cells in latent forms. Several factors can induce cells to activate latent TGFβ. Activation of latent TGFβ has been demonstrated by several cell types, such as macrophages treated with γ-interferon, IgG, or lipopolysaccharide [Twardzik et al., 1990], mesenchymal cells treated with glucocorticoids [Rowley, 1992], osteoclasts treated with retinol [Oreffo et al., 1989], endothelial cells treated with fibroblast growth factor [Flaumenhaft et al., 1992] and osteoblast-like cells treated with parathyroid hormone [Yee et al., 1993]. Endothelial cells cocultured with pericytes or smooth muscle cells will also activate latent TGFβ [Antonelli-Orlidge et al., 1989]. In these cocultures, activation was shown to be due to the generation of plasmin [Sato and Rifkin, 1989]. Plasmin has been shown to digest the TGFβ precursor or latency associated peptide which results in release of mature or active TGFβ [Lyons et al., 1990]. Osteoclasts appear to activate latent TGFβ by an unknown but different mechanism than plasmin activation [Oursler et al., 1994].

Once TGFβ is released from its latent complex it can bind to receptors or it can bind to other matrix proteins. The matrix proteoglycan, decorin, can bind to TGFβ, thereby inactivating the molecule [Border and Ruoslahti, 1992]. Decorin has been shown to prevent TGFβ-induced glomerulonephritis [Border et al., 1992], whereas thrombospondin appears to bind to TGFβ and maintain its activity [Schultzcherry and Murphyullrich, 1993]. Alpha-$_2$ macroglobulin appears to covalently bind TGFβ, thereby inactivating it, and may act as a scavenger to remove TGFβ from the circulation [O'Connor-McCourt and Wakefield, 1987]. Therefore, there are many levels at which TGFβ actions can be controlled, including secretion, storage, and activation of latent forms. Once activated further control can be exerted by blocking of binding to receptors, stabilizing activity, and removal/disposal of the active molecule.

BIOLOGICAL FUNCTIONS OF TGFβ

Most in vitro studies show that TGFβ is obviously very important in matrix formation, as it stimulates numerous matrix proteins such as collagen, laminin, fibronectin, and receptors as-

sociated with these proteins. In fact, one of its earliest names was cartilage inducing factor [Seyedin et al., 1985]. TGFβ has been gaining the reputation of being a "bad" factor for several tissues. For example, excess TGFβ appears to be responsible for glomerulofibrosis in the kidney [Border et al., 1992]. Parasites can escape the immune system by activating latent TGFβ [Barral-Netto et al., 1992]. However, TGFβ may have therapeutic uses in wound healing [Amento and Beck, 1991], and administration of TGFβ in the model of experimental allergic encephalomyelitis reduces incidence and severity of disease [Johns and Sriram, 1993]. These studies emphasize the necessity of tightly regulating the activation of latent TGFβ or the activity of active TGFβ.

For many cell types TGFβ is a reversible inhibitor of growth. Many cells are retained in G_o/G_i by TGFβ and cannot progress into S phase possibly because of interruption of phosphorylation of pRB, a nuclear 105 kD protein, known as a tumor suppressor [Moses et al., 1990]. When TGFβ does stimulate proliferation, this is usually accomplished by production of a secondary growth factor. Inhibition of growth usually occurs within hours and stimulation greater than 24 h.

Studies using transgenic "knock-out" mice deficient in TGFβ1 clearly emphasized the important role of TGFβ1 in immunosuppression; however, these studies did not answer the question of how important TGFβ is in bone formation. The animals began to die from massive macrophage infiltration only after weaning [Shull et al., 1992; Kulkarni et al., 1993]. This is because the fetuses were exposed to TGFβ1 through placental transfer and the neonates were exposed to TGFβ through mother's milk. Death occurred before changes in bone could be observed.

It has been suggested that TGFβ acts as a "coupling factor" in bone which couples resorption to formation [for review see Bonewald and Mundy, 1990] (see Fig. 2). Since TGFβ is released by resorbing osteoclasts and in vivo injections of TGFβ can induce new bone formation [Pheilschifter and Mundy, 1987; Noda and Camilliere, 1989; Marcelli et al., 1990], TGFβ appears to have important biological functions in bone. Bone is the largest source of TGFβ in the body. High concentrations of TGFβ inhibit mature osteoclastic bone resorption, and low concentrations inhibit osteoclast formation (see Fig. 2). Therefore, TGFβ released by resorbing osteoclasts could act to both stimulate new bone formation and limit the extent of further bone resorption.

As two latent forms of TGFβ are produced by bone cells, we have suggested that the 100 kD form may be more readily activated and that the form containing the binding protein may be targeted for matrix storage [Dallas et al., 1994]. Recently, the precursor or latency associated protein has been shown to bind the latent complex to smooth muscle cells [Sato et al., 1993]. It is not known if this binding occurs via a receptor or other types of binding, but it does not appear to bind through mannose-6 phosphate or RGD sequence binding. These data support the hypothesis that the 100 kD form may be targeted to cells for activation.

Our preliminary data treating cultures of fetal rat calvarial cells with antibody specific for TGFβ binding protein show inhibition of nodule formation. The data suggest that the latent complex containing the binding protein may not only be a storage form for TGFβ in bone matrix but may play a role in directing cellular migration towards nodule formation. Alternatively, the antibody to the binding protein could be preventing the complex from forming the fibrillar strands in the matrix, thus enabling more activation to occur, and therefore the active TGFβ could be inhibiting nodule formation [Harris et al., in press]. These hypotheses are now under investigation.

STIMULATION OF BONE FORMATION

In vivo injection of TGFβ into bone sites leads to new bone formation with or without a cartilaginous intermediate depending on the site of injection. A single injection of TGFβ will induce complete healing of a nonhealing skull defect [Beck et al., 1991]. The key word is *single* injection. This study by Beck and coworkers shows that TGFβ initiates a cascade of events leading to new bone formation. They postulate that TGFβ is stimulating the recruitment and proliferation of osteoblasts to the defect site. They suggested that increased numbers of osteoblasts were responsible for new bone formation and that matrix secretion by osteoblasts was not affected by exogenous TGFβ. TGFβ is a potent chemoattractant for osteoblasts [Pfeilshifter et al., 1990]. Human bone marrow contains cells which form colonies and respond to TGFβ with an increase in alkaline phosphatase, osteonec-

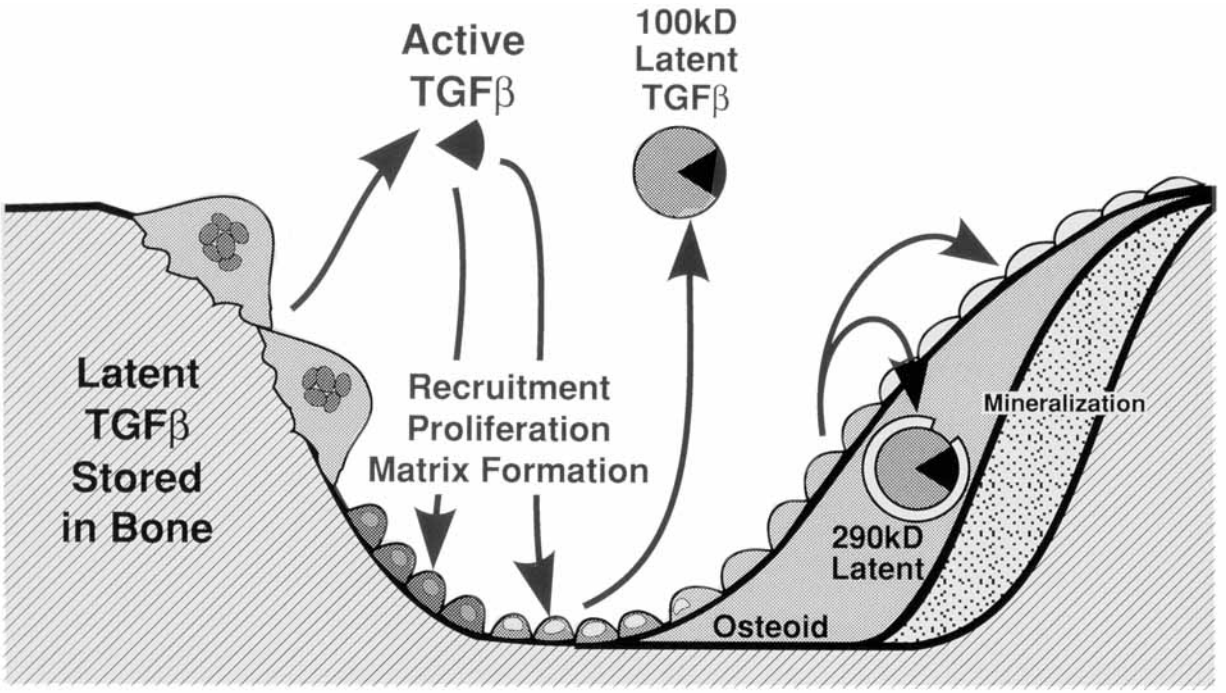

Fig. 2. A drawing depicting the potential roles of active TGFβ, the 100 kD latent complex lacking the binding protein, and the latent complex containing the binding protein. Latent TGFβ is stored in bone where it is released in an active form by resorbing osteoclasts. Active TGFβ is responsible for the recruitment and proliferation of osteoblasts. These osteoblasts make two forms of latent but no active TGFβ. The 100 kD form may be targeted to cells for cellular activation, whereas the latent complex containing the binding protein forms fibrillar strands in the bone matrix, either for storage or for forming the proper matrix for bone formation.

tin, and osteocalcin [Long et al., 1990]. These could be the cells recruited by in vivo injections of TGFβ. TGFβ could be indirectly stimulating proliferation of the recruited osteoblasts and could possibly play a role in differentiation and matrix production by these cells.

Others have suggested that TGFβ stimulates osteogenesis by acting on committed periosteal cells. These already stationary committed cells would be responsible for matrix formation, not recruited precursors. These questions concerning the target cell of TGFβ for in vivo bone formation remain to be resolved.

INHIBITION OF MINERALIZATION

Kato and coworkers [1988] suggested that TGFβ could inhibit mineralization. They reported that TGFβ suppresses the mineralization-related phenotype in rabbit chondrocyte tube cultures. This observation was followed by work of Antosz and coworkers showing that TGFβ inhibited nodule formation and consequently mineralization in fetal rat calvarial cell cultures [Antosz et al., 1989]. At the same time, other publications showed that in vivo injections of TGFβ stimulated new bone formation. Investigators in the bone field were faced with the paradox of TGFβ stimulating new bone in vivo but inhibiting new bone formation in vitro. When in vitro results do not reflect in vivo observations, this suggests that the in vitro assay is not representative of the events occurring in vivo or the target cell used in vitro is not the correct cell.

A wide variety of osteoblast-like cell lines have been used to test the effects of TGFβ on parameters which are representative of the osteoblast phenotype. These osteoblast characteristics include regulation of alkaline phosphatase and osteocalcin, the cAMP response to parathyroid hormone, production of collagen and other matrix proteins, and cellular proliferation. Cell lines tested included ROS 17/2.8, UMR-106, MC3T3E1, SaOS-2, MG-63, and others [for review see Centrella et al., 1991]. A variety of effects was observed, ranging from stimulation to inhibition depending on culture conditions. Criticisms were raised concerning the transformed phenotype of cell lines. When normal osteoblast populations were examined, specifi-

cally the fetal rat calvarial cells and isolated explant bone cells, again the effects of TGFβ were different depending on the cell population and assay conditions. Determining the mechanism whereby TGFβ stimulates new bone formation became difficult and unclear. A few cell lines would closely mimic normal cells; for example, MC3T3 closely mimic isolated fetal rat calvaria cells by responding to TGFβ with a decrease in alkaline phosphatase [Noda and Rodan, 1986], and MG-63 cells respond the TGFβ and $1,25(OH)_2D_3$ in the same manner as human explant bone cells [Bonewald et al., 1992; Wergedal et al., 1992]. Therefore, though transformed, a number of the osteoblast-like cell lines will respond to TGFβ in the same manner as normal osteoblasts.

It is important to decide what in vivo situation a particular in vitro assay system represents. The osteoblasts isolated from fetal rat calvaria are already "programmed" to form bone. These cells in culture form nodules with the characteristics of bone and will do so in the presence of 5% serum. TGFβ inhibits proliferation of these cells (growth suppression) and inhibits formation of nodules [Antosz et al., 1989] while stimulating matrix formation. However, messenger RNA for type I collagen, alkaline phosphatase, osteopontin, and osteocalcin is inhibited when compared to control cultures [Harris et al., in press; Breen et al., in press]. The effects of TGFβ are reversible in these cultures, unlike the effects of other inhibitors. Very recently, others using different systems such as differentiation of mesenchymal stem cells [Iwasaki et al., 1993] and mineralizing bone organ cultures [Chen and Bates, 1993] have come to the same conclusion that TGFβ inhibits calcification.

A potential mechanism for the inhibitory effect of TGFβ on mineralization could be by increasing ectonucleoside triphosphate pyrophosphatase activity in the bone microenvironment. This enzyme catalyzes the hydrolysis of nucleoside triphosphates to nucleoside monophosphates and inorganic pyrophosphate and may be a major source of inorganic pyrophosphatase in bone. Inorganic pyrophosphatase may enhance the initial precipitation of amorphous calcium phosphate but retard its transformation to hydroxyapatite. Support for an inhibitory role of the enzyme in mineralization comes from a rare genetic condition of hypophosphatasia. In this condition reduced clearance of inorganic pyrophosphate is associated with defective mineralization. Chondrocytes, osteoblasts, and matrix vesicles from these cells make ectonucleoside triphosphate pyrophosphatase.

Alkaline phosphatase appears to catalyze the breakdown of inorganic pyrophosphate. There is considerable evidence that alkaline phosphatase plays a key role in the formation and calcification of bone. The lack of alkaline phosphatase in hypophosphatasia may account for the reduced clearance of extracellular inorganic pyrophosphate. Oyajobi and coworkers have shown that TGFβ stimulates the production of ectonucleoside triphosphate pyrophosphatase in human explant bone cells [Oyajobi et al., 1994] while inhibiting the expression of alkaline phosphatase. This combined effect may be a major mechanism whereby TGFβ inhibits mineralization. Interestingly, like TGFβ, $1,25(OH)_2D_3$ also increases ectopyrophosphatase activity in human explant bone cells [Oyajobi et al., 1989], and, like TGFβ, $1,25(OH)_2D_3$ inhibits nodule formation by fetal rat calvarial cells [Ishida et al., 1993]. It remains to be determined whether TGFβ and $1,25(OH)_2D_3$ also increase ectopyrophosphatase in fetal rat calvarial cells.

Another marker of mineralization that is inhibited by TGFβ is osteocalcin. Osteocalcin is the most abundant noncollagenous protein in bone [Poser et al., 1980] and is a specific marker for bone and cartilage cells. $1,25(OH)_2D_3$ stimulates osteocalcin in human explant bone cells, fetal rat calvarial cells, ROS 17/2.8 rat osteosarcoma cells, and MG-63 human osteosarcoma cells [Owen et al., 1991; Bortell et al., 1993]. TGFβ is a powerful inhibitor of this protein [Noda, 1989]. TGFβ inhibits osteocalcin production by fetal rat calvarial cells and ROS 17/2.8 cells and will inhibit $1,25D_3$ induced osteocalcin production in MG-63 cells [Bonewald et al., 1992]. Blocking the production of this bone specific marker is probably not the mechanism whereby TGFβ inhibits mineralization but the result of this inhibition.

RELATIONSHIP OF TGFβ TO BONE MORPHOGENETIC PROTEIN (BMP)

A factor that can induce mineralization is bone morphogenetic protein-2 (BMP-2) (see review by Ghosh-Choudhury et al.) TGFβ will only induce new bone formation when injected in close proximity to bone. The bone morphogenetic proteins or BMPs will produce bone when injected into ectopic sites. TGFβ and BMP-2 may therefore complement each other by affect-

ing different phases of new bone development or different phases of the bone formation cascade. TGFβ may predominately initiate bone formation during injury or normal bone remodeling by recruitment and proliferation of osteoblast precursors, whereas BMP-2 appears to be very important in inducing differentiation in multipotential progenitor cells. In vitro BMP-2 usually stimulates the expression of markers of the osteoblast phenotype, such as alkaline phosphatase, in a number of osteoblast-like cell lines and normal osteoblasts. A mesenchymal progenitor cell, C3H10T½, when transfected with BMP-2 expresses the osteoblast phenotype and will mineralize in culture [Ahrens et al., 1993]. The target cells for TGFβ and BMP that are responsible for bone formation may be quite different (see Table I).

Although BMPs are members of the extended TGFβ superfamily due to structural similarities, BMP-2 has very different effects on bone cells when compared to TGFβ. BMP-2 will stimulate alkaline phosphatase expression in most osteoblast and osteoblast-like cells and will accelerate nodule formation in fetal rat calvarial cultures. In contrast, TGFβ inhibits alkaline phosphatase and nodule formation by fetal rat calvarial cells [Harris et al., in press]. Whereas TGFβ will inhibit osteocalcin, BMP-2 stimulates the production of this marker of mineralization. TGFβ actually inhibits BMP expression in fetal rat calvarial cell cultures [Harris et al., in press], whereas BMP-2 has been shown to stimulate mRNA for TGFβ1 in an osteoblast-like cell line [Zheng et al., in press]. Since the TGFβs are synthesized as latent complexes, it is assumed that BMP-2 is inducing the production of latent TGFβ which could then be activated during the endochondral ossification process. BMPs and TGFβ are both involved in the bone formation process. BMPs may play a greater role in development and fetal bone formation by induction of the osteoblast phenotype leading to mineralization, whereas the role of TGFβ in adult bone remodeling and bone repair may be more relevant and important.

TABLE I. Characteristics of BMP-2 and TGFβ1

Characteristic	BMP 2	TGFβ1
Ectopic bone formation	Yes	No
New bone formation	?	Yes
Alkaline phosphatase	Promotes in most osteoblast-like cells	Promotes in some osteoblast-like cells
Osteocalcin production	Promotes	Inhibits
Mineralization	Promotes	Inhibits
Amount in bone	1–2 ng/gm	450 ng/gm
Latent forms	?	Yes
Mature form	30 kD homodimer glycosylated 7-disulfide bonds	25 kD homodimer nonglycosylated 9 disulfide bonds
Precursor	Monomer	Latency associated homodimer
Binding proteins	?	Yes
Target cell	Multipotent progenitor?	Osteoblast or osteoblast precursor?
Regulation of mRNA	Stimulates TGFβ mRNA	Inhibits BMP-2 mRNA

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