Transforming growth factor beta (TGF-β) in bone remodelling

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The current concepts of the role of transforming growth-factor beta (TGF-β) with regard to bone metabolism and fracture repair are reviewed. The sources, types, chemistry, cellular activity and the clinical applications of TGF-β have been discussed. In low concentrations, this growth factor has significant effects on bone remodelling. The use of TGF-β for practical therapeutic purpose remains an exciting challenge.

BONE differs from other tissues not only in physiochemical structure but also in its extraordinary capacity for growth, continuous internal remodelling and regeneration throughout postfetal life. Bone formation is a complex process regulated by diet, vitamins, hormones and growth factors[1]. Growth factors are polypeptides that increase cell replication and have important effects on differentiated cell function. Growth factors were initially considered as systemic agents, but current evidence indicates that they act primarily as local regulators of cell growth[2]. The transforming growth factor-β family of proteins, which comprises polypeptide growth factors that have diverse effects on the growth, differentiation, and function of cells, is receiving considerable attention for potential clinical applications and is likely of physiological and surgical significance.

Transforming growth factors

It is one of the most important growth-promoting osteoinductive substances, which have been identified at the site of fractures and other places. Transforming growth factors (TGFs) have been categorized into TGF-α (alpha) and TGF-β (beta). TGF-α has not been isolated from bone tissue and cannot be considered a local regulator of bone remodelling, although it is mitogenic for bone cells and stimulates bone resorption. TGF-α shared amino acid sequence, receptor-binding and functional activity with epidermal growth factors but had only weak transforming growth factors activity[3]. Furthermore, TGF-β by itself was completely ineffective, but together with TGF-α it potently induced growth of colonies of cell-line[3].

TGF-β is a multifunctional growth factor that has been shown to mediate normal cellular physiology and tissue embryogenesis and to participate in a variety of responses associated with inflammation and tissue repair[4].

Transforming growth factor-beta

Sources

TGF-β was originally purified from human platelets[5], human placenta[6] and bovine kidney[7]. The largest source of TGF-β in the body is the extracellular matrix of bone and platelets may represent the second largest reservoir for the peptide[8].

Chemistry and types

TGF-β is a highly stable molecule that consists of two identical chains, each containing 112 amino acids. TGF-βs are a family of polypeptide growth factors encoded by closely related genes. There are at least five TGF-βs: TGF-β_1 to TGF-β_5. TGF-β_1, β_2 and β_3 have been found in many species, including humans; β_4 has been found in chickens and β_5 has been found in amphibians[9]. All share 64–82% similarity in their amino acid sequence[10]. Human platelets contain a single form of TGF-β composed of two identical polypeptides constituting a dimer of relative molecular mass (Mr) 25,000 (ref. 11).

A very high level (more than 95%) of sequence homology for a single isoform of transforming growth factor-β among many species indicates that these five isoforms are not simply species-specific variants[12]. All the five isoforms might be expressed within a single species, in which all or a subset of the transforming growth factors βs may be synthesized by particular tissue at specific stages of development, or, after appropriate stimulation. A number of biochemicals featured are shared by most of these closely-related proteins[4]. The mature TGF-β subunit contains two identical chains, each containing 112 amino-acids (except TGF-β_4) and 9 cystine residues whose locations are conserved in all five isoforms[13]. The active proteins are approximately 25 kDa and are composed of two, usually identical, disulphide-linked subunits[14]. Physical characteristics of the mature polypeptide subunits of TGF-βs are listed in Table 1.

Table 1. Physical characteristics of the mature polypeptide subunits of TGF-βs[15]

Protein	Amino acids per subunit	Molecular weight (Mr) (Da)	Conserved cystein
TGF-β_1	112	12,500	9
TGF-β_2	112	12,500	9
TGF-β_3	112	12,500	9
TGF-β_4	114	12,900	9
TGF-β_5	112	12,500	9

TGF-β is synthesized in precursor form that requires proteolytic processing. It is released from the cell in an inactive high molecular weight complex composed of the processed dimer in combination with amino terminal fragments of each of the two TGF-β monomeric precursors and a third uncharacterized molecule. The physiological mechanism by which TGF-β is released from the inactive complex is uncertain but may require specific enzyme activation[16,17].

Cellular activity

Transforming growth factor-β has a broad range of cellular activities, including the control of the proliferation and expression of the differentiated phenotype of several types of cells specific to the skeleton, among them the pleuripotent undifferentiated mesenchymal cells (pericytes) for chondrocytes, osteoblasts and osteoclasts[18]. *In vivo* and *in vitro* studies have demonstrated the synthesis of TGF-β by chondrocytes and osteoblasts and their expression is increased in fracture callus and specifically regulates bone-associated activities that may be important for the initiation of repair of fracture[19]. Studies on the potential role of transforming growth factor β in soft tissue wound healing have shown that this molecule is released from degranulating platelets at the site of an injury, possibly to initiate a cascade of reparative events[20].

Histologically, TGF-β increases nuclear 34-thymidine labelling in the osteoblast precursor cell zone[21]. Studies with isolated bone cells show that TGF-β is a potent regulator of osteoblastic cell activity and on a molar basis TGF-β is one of the most effective mitogens so far described for osteoblast-enriched cultures from fetal bone[22,23].

Studies have also shown that TGF-β is the most potent chemotactic factor released from macrophages[8]. In addition to attracting macrophages to sites of injury, this peptide activates the synthesis of other growth factors and deactivates the production of hydrogen peroxide by macrophages, so that young tissues are not destroyed[24]. Moreover, TGF-β has been shown to stimulate fibroblast-directed tissue repair by upregulating the production of extracellular matrix components, such as collagen, fibronectin and proteoglycan, by upregulating the expression of cellular integrin receptors for extracellular matrix proteins; and by inhibiting the action of proteolytic enzymes that could destroy newly-formed osseous tissue[16].

TGF-β produce pleuripotent and biphasic changes in the biochemical activities of cells at particular stages of differentiation within the osteoblast lineage[4]. To date, most studies to evaluate the effectiveness of TGF-βs in bone are performed with the prototypical isoform of TGF-β_1 derived from blood platelets, but TGF-β_1, β_2, β_3 each have identical qualitative effect in osteoblast enriched cell cultures[22].

All the three isoforms potently increase synthesis of bone DNA at low concentration but have reduced mitogenic activity at higher levels[4]. These isoforms also enhance synthesis of collagen and non-collagen protein and decrease activity of alkaline phosphate in osteoblast enriched cultures, but TGF-β_3 appears to be 3–10 times more potent than TGF-β_1 and β_2 (ref. 25). Similarly, the TGF-βs enhance replication of cells and production of bone matrix in cultures of intact bone *in vitro*. In most instances, the TGF-βs enhance synthesis of type-1 collagen and non-collagen protein in bone cells[26]. The mitotic response to TGF-β is biphasic; low concentrations (below 100 rM) are stimulatory, whereas higher levels produce less of a stimulatory effect. At higher, less mitogenic concentrations, TGF-β alters expression of various activities associated with the osteoblast phenotype in different ways; under these conditions, TGF-β decreases alkaline phosphatase activity and similar to its effects in a number of other connective tissue systems, enhances the synthesis of type I collagen, the major organic element in the bone matrix[22,27]. Furthermore, TGF-β_1 and β_2 enhance chemotaxis of osteoblasts[28], suggesting an additional role involving the recruitment of differentiated osteoblasts that may be necessary for new bone formation and fracture repair.

Recent studies in osteoblast-enriched cultures have demonstrated that TGF-β_1 increases synthesis of type 1 collagen polypeptide in the absence of mRNA transcription and decreases non-collagen in the bone matrix as a result of the action of TGF-β probably occurs by multiple transcriptional, post-transcriptional and post-translational events[4]. Some other activities of TGF-βs associated with the function of osteoblasts have also been examined. TGF-β_1 and β_2 increase the transcriptional and polypeptide levels of osteopontin, a protein in the bone matrix that is thought to be important in adhesiveness of bone cells[29]. In osteosarcoma cultures, TGF-β enhances Type 1 collagen synthesis as well as the production of a variety of bone matrix-associated polypeptides such as osteonectin, osteopontin and alkaline phosphatase[29–33], but decreases osteocalcin synthesis[34]. These findings indicate that TGF-β enhanced bone matrix accumulation and consequently the maintenance of

bone mass. In general, the stimulatory influence of TGF-β on bone matrix protein synthesis may be attributed to effect at the transcriptional level[22,27,31].

However, a direct relationship between the influence of TGF-β on type I collagen mRNA and polypeptide levels is not observed in isolated bone cells, suggesting that TGF-β has an additional supportive role in increasing bone matrix formation[22]. The protein in the bone matrix termed osteonectin, which may be involved in the deposition of type-1 collagen and the transition between cartilage and bone, was increased by TGF-β_1, in an *in vitro* model that was used to study repair of fracture[35].

Much less information is presently available regarding direct effects of the TGF-βs on osteoclasts or osteoclasts precursors. Recently, however, biphasic effects on the development of osteoclast-like cells have been reported, in which low concentrations were stimulatory and higher concentrations were inhibitory. TGF-β_1 induces resorption of bone on synthesis of prostaglandin E_2 (ref. 36).

In fetal rat long bones, TGF-β decreases bone resorption[37], and this effect may be related to the ability of TGF-β to inhibit the formation of osteoclast-like cells *in vitro*[38]. This difference may be a significant distinction in bone growth and development between the fetus and the new born; within very early stages of bone formation, matrix formation may be more important to the organism than bone remodelling. It is therefore suggested that TGF-β has an additional, indirect role in increasing or maintaining body mass[37].

With regard to formation of cartilage, the TGF-βs appear to increase differentiation of mesenchymal cells, production of proteoglycans, and replication of chondroblasts[39]. In contrast, these agents usually decrease the proliferation and function of differentiated cells by more mature chondrocytes. For example, TGF-βs decrease the activity of alkaline phosphatases[27]. The highest levels of TGF-β mRNA were associated with osteoblasts in developing bone, whereas TGF-β transcripts decreased in chondroblasts/chondrocytes with increased type II collagen expression[40].

In general, it seems that one important role for the TGF-βs is to include developmental transitions in cells that are involved in formation of endochondral bone at particular stages of differentiation. The predominant trend of these effects appears to be toward eventual *de novo* formation of bone[4].

Clinical applications

The use of TGF-βs to enhance wound healing was first investigated by Mustoe *et al.*[41], who applied this peptide directly to and immediately after the creation of linear incisions through the dorsal skin of rats. These investigators demonstrated a 220% increase in the maximum strength at the site of the wound after five days and an acceleration in the rate of healing by at least three days compared with that in the control animals.

It is also suggested that this peptide molecule has the capacity to stimulate both intramembranous and endochondral formation of bone[35]. Noda and Camilliere[42] studied the effect of daily injection of 1 µg of TGF-β directly into the periosteum of the parietal bones of neonatal rats. They showed that (i) the thickness of the treated parietal bones increases approximately twofold, (ii) this effect was localized to the site of the injection, (iii) no changes were observed in the contralateral bones or at distant skeletal sites.

Joyce *et al.*[19] performed a similar study in the femora of newly-born rats and found that on daily injection of either TGF-β_1 or β_2 into the subperiosteal region of the femora, mesenchymal precursor cells in the periosteum were stimulated to proliferate and differentiate much the same as is observed during the embryological formation of bone and early fracture healing. After cessation of the injection, endochondral ossification also occurred and this resulted in the replacement of cartilage with bone. In addition, it was shown that injection of TGF-β_2 stimulated the synthesis of TGF-β_1 in chondrocytes and osteoblasts, suggesting autoregulation of this peptide[9,19].

It has been demonstrated that a single injection of 25–100 nanogram (ng) of recombinant human TGF-β_1 adjacent to the ear cartilage of rabbits stimulated the formation of bone after 21 days[43]. In a subsequent investigation, they showed that exogenously applied recombinant human TGF-β stimulated the recruitment and proliferation of osteoblasts in critically-sized defects in the skull of rabbits. By assessing the temporal dynamics of the formation of bone in these defects, they demonstrated the potent osteoinductive activity of this peptide and its potential therapeutic applications for non-healing osseous defects[44].

The function of TGF-β, both *in vitro* and *in vivo*, can be highly variable. In some instances this growth factor inhibits cell growth and matrix synthesis, whereas in others it stimulates these processes. These different functions depend on the target cell, the presence or absence of other cytokines and the dose[45]. In their study, Sumner *et al.*[46] suggested that the response to treatment with TGF-β was dose dependent, with the lower dose being more effective for enhancing bone ingrowth, whereas, high dose of TGF-β inhibited mineralization. Synthesis of osteocalcin, a calcium binding protein important in mineralization[47] is inhibited, possibly due to this growth factor.

A similar observation was also reported by Nielsen *et al.*[48], who injected TGF-β, 4 to 40 ng in every alternate day for 40 days after creation of tibial fractures in rats. Mechanical testing showed that TGF-β induced a dose-dependent increase of the callus at the site of the fracture.

Enhancement of bone ingrowth with TGF-β was also evaluated in the canine model[46]. Sumner *et al.*[46] observed that treatment of an implant (titanium fibre metal-coated rod) with a combination of a hydroxyapatite–tricalcium phosphate coating and TGF-β may result in better bone ingrowth than that obtainable from grafting of the gap with autogenous cancellous bone. The amount of new bone formation in the three millmeter gaps adjacent to the treated implants was twice that in the gaps of the paired controls, regardless of the dose. The differences between the treated and control implants with regard to architecture of the new bone in the gap indicate that the mechanism of action of TGF-β_1 may include both proliferation of osteoprogenitor cells and production of matrix by committed osteoblasts[46].

Histological study with two *in vivo* models of osteogenesis have also demonstrated an increase in net formation of bone by TGF-β_1 and β_1, when the agents were tested either by direct application to the tissue or by subcutaneous injection[42].

Summary

The transforming growth factor-βs are polypeptide growth factors encoded by a family of closely-related genes that are expressed in numerous tissues and species. Bone was one of the first tissues in which locally-produced molecules with TGF-β like activity appeared to regulate normal cellular function, and the skeletal matrix probably comprises the largest reservoir of TGF-βs. *In vitro* and *in vivo* studies have indicated that TGF-βs can have either stimulatory or inhibitory effects on replication, lineage development, and differentiated phenotypic function in many types of skeletal tissue cells[4].

The effect may be biphasic in the sense that low concentrations of TGF-βs stimulate proliferations of cells, whereas high concentrations inhibit proliferation. Even within the same cell lineage, opposite effects have been noted with different concentrations of TGF-βs, or with skeletal cells at different development stages[4].

In foetal tissue, TGF-β stimulates differentiation of mesenchymal cells, proliferation of osteoblasts and synthesis of bone matrix, but it may also induce maturation into and through the osteoblast, chondrocytes and osteoclast lineages[4].

Most evidence to date leads to the conclusion that the TGF-βs have an important role in the formation of bone and this has tremendous potential in therapeutic application for non-healing osseous defects and in different types of fracture healing.

Finally, continued *in vivo* and *in vitro* studies of these molecules are needed in order to evaluate their effective doses, appropriate duration of treatment, tissue specificity, receptor-binding, intracellular signalling and the regulation of their activities by other local and systemic factors.

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RESEARCH ACCOUNT

Bile acids in asymmetric synthesis and molecular recognition

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This account summarizes the progress made in our laboratory towards the development of new uses of naturally occurring bile acids. Applications in Asymmetric Synthesis (*intra*molecular coupling, and *inter*molecular reactions) and Molecular Recognition are described with suitable examples.

NATURE is asymmetric and almost all chiral molecules present in nature are homochiral (i.e. they exist in only one of the two possible enantiomeric forms). Organic chemists have always been fascinated by the possibility of constructing natural products by total synthesis. This approach was very successful till the sixties for constructing racemic modifications of chiral natural products. The methodology for synthesizing only one (the naturally occurring form) enantiomer was not present in the organic chemists' 'toolbox' until the seventies. During the past two decades, however, asymmetric synthesis of a variety of molecules has been accomplished by chemists using an assortment of techniques. In most of these examples, the inherent chirality of natural products (such as terpenes, sugars, amino acids etc.) has played important role.

The 'lock and key' concept of Emil Fischer for explaining the specificity of enzymatic action has been known for a century. Deliberate attempts to mimic such biological processes in the laboratory with small organic molecules, however, started much later. Early work with cyclodextrins and subsequently with crown ethers were forerunner to a new area of research towards the design, synthesis, evaluation and applications of synthetic molecular receptors. Carefully designed studies on molecular receptors have provided new insight into molecular interactions. In addition, recent research has shown that many molecular receptors can be designed to have tailor-made properties and hence can be used as a variety of molecular devices including molecular sensors (see, for example, ref. 15).

1. Bile acids: their properties

Bile acids, secreted by the liver, are important metabolites of cholesterol (Figure 1). These compounds are sometimes found in the form of conjugates, specially with glycine and taurine. Most bile acids are