The cell and molecular biology of bone fracture repair: role of the transforming growth factor-β1 in activation reparative osteogenesis (review)

S. Sagalovsky, M. Schonert

Department of Orthopedic Median Clinic, Bad Lausick. Germany

This paper describes the cellular and molecular mechanisms underlying in the basis of bone fractures healing. It is shown that there are two types of fracture healing. In the case of the primary type bone tissue directly form in zone of damage. In the case of instability of the bone fragments and considerable distance between them the process of restoration of the integrity of the bone goes through several stages including inflammation, migration and proliferation of cells, the formation of tissue-specific structures and the restructuring of the regenerate. At the same time at different stages certain factors affecting the proliferation, differentiation of osteogenic cells and synthesis of extracellular matrix release. Growth factors control the various stages of reparative osteogenesis. In recent years the regulatory role of transforming growth factor beta-1 (TGF-β1) and belonging to his family of bone morphogenetic protein (BMP) in the formation and development of bone regeneration was confirmed. According to modern scientific ideas BMP act as primary activators of differentiation of osteogenic progenitor cells, and mesenchymal cells, and are involved in the processes of bone remodeling and fracture healing. Initially it was thought that TGF-β1 stimulates the proliferation of undifferentiated mesenchymal cells and chondrocytes as well as the production of extracellular matrix during chondrogenesis and enchondral osteogenesis. As shown in recent studies optimizing effect of TGF-β1 on the processes of fracture healing is dose-dependent and requires its constant high concentration. In-depth understanding of the mechanisms of development reparative osteogenesis allows to develop a new strategy for optimization of the process of restoring the integrity of bone broken due to injury. Key words: osteogenesis, bone fracture healing, transforming growth factor β-1, bone morphogenic protein.

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Bone fractures are a major public health concern due to their negative impact on health outcomes, quality of life, and costs [1]. Worldwide bone fractures projection has suggested approximately 16.2 million bone fractures per year. The number is assumed to increase to 36.5 million [2] or even to 52.3 million [3, 4] in 2050. Bone fracture repair is an extremely complex process which depends on the coordinated action of several cells lineages on a cascade of biological events, and has always been a major medical concern. The process of fracture repair has been described in detail in many studies [5–8]. Recent work has focused on the mechanisms by which growth and differentiation factors regulate the fracture healing process. Rapid progress in bone cellular and molecular biology has led to the identification of many signaling molecules associated with the formation skeletal tissues, including members of transforming growth factor-ß (TGF-ß) superfamily [9].

**General fracture healing**

Two different types of fracture healing are known to repair a fractured bone. Primary fracture healing is the direct growth of bone at the fracture site. This type of healing occurs, if the fracture is stable and the gap is very small. In case of instability and moderate gap size, secondary healing occurs. This type of fracture healing can be divided into several stages including inflammation, soft callus formation, hard callus formation and remodeling [10]. This report deals with secondary fracture healing, which is clinically more relevant. The histologic progression of secondary fracture [11] repair can easily be divided into four distinct stages, each characterized by different cellular features and an extracellular matrix (fig. 1). The first stage after the injury is inflammation (fig. 1, a) [12, 13]. During the injury, blood vessels are ruptured, which leads to extensive hemorrhage and the release of several signaling factors [8, 14]. The result is a hematoma (blood clot) surrounding the fracture. In this early stage the released signaling factors are mainly cytokines and growth factors, including transforming growth factor beta-1 (TGF-ß1) and bone morphogenetic proteins (BMPs) [6, 7]. Relatively little is understood of the precise working of the regulating function of these growth factors, but known from embryonic bone development research and also from in vitro studies, these factors are likely to play important roles in fracture healing [15, 16]. The adjacent soft tissue undergoes necrosis and the rupture of blood vessels causes the bone cells to die. Macrophages digest the dead tissue and the hematoma is replaced by granulation tissue. Granulation tissue is the tissue in the initial, soft callus. From a mechanical point of view, a callus stabilizes the fracture by increasing the cross-section diameter [17]. Because granulation tissue is stiffer compared to the hematoma, the callus increases its stiffness as well.

During the second stage mesenchymal cells migrate into the callus (fig. 1, b). It is assumed that these cells originate from the periosteum, endosteum, marrow and surrounding soft tissue [6, 18, 19]. Mesenchymal cells proliferate and differentiate into fibroblasts, chondrocytes or osteoblasts depending on the biological and mechanical conditions. These cells produce fibrous tissue, cartilage and bone matrix, respectively [20]. More growth factors, including TGF-ß1 and BMPs, are released and at the end of this stage the release of angiogenic factors (vascular endothelial growth factor) increased [8, 16, 21]. At each side of the fracture gap near the bone tissue, the mesenchymal cells differentiate into osteoblasts producing intramembranous woven bone. Further away from the bone tissue towards the centre of the callus, mesenchymal cells differentiate into either fibroblasts or chondrocytes. Because of this, the callus is gradually stabilized.

In the third stage of the healing process, both intramembranous and endochondral ossification takes place (fig. 1, c). The fibroblasts and the fibrous tissue are replaced by chondrocytes and cartilage. This is coupled with the increased release of TGF-ß and BMPs. The release of angiogenic factors increases as well, resulting in the growth of blood vessels inside the bone, which are necessary for replacement of cartilage by bone. These new vessels can only grow and sustain if the mechanical conditions allow it [14, 22]. Large interfragmentary movements may rupture or collapse the new blood vessels and thus preventing them from supplying the new bone with their metabolic needs. At either side of the callus an osseous bridge is formed, making the callus mechanically more stable. A creeping substitution of bone towards the gap occurs. After the centre of the callus has ossified, the healing is completed. In the final stage bone forms from cartilage in the soft callus by a process that appears similar to bone formation in the growth plate. Chondrocytes adjacent to the subperiosteal bone hypertrophy, the cartilage extracellular matrix calcifies, and capillaries from adjacent bone invade the calcified cartilage (fig. 1, d). Osteoblasts follow capillary ingrowths and synthesize osteoid on the calcified cartilage, forming primary spongiosa with the distinctive «mixed spicule» that contains both bone and cartilage [23]. This process continues until all cartilage in the soft callus is replaced by bone, «bridging» the fracture gap, when bone bridging has occurred, mechanical stability is restored, and remodeling of new bone and underlying bone cortex restores the normal bone architecture [24]. While each of the four stages of fracture repair has distinct histological
features, they share several underlying cellular events that are subject to regulation. These events include cell proliferation and differentiation, chemotaxis, and the synthesis of extracellular matrix. The repair of fractures is believed to be regulated by both systemic and local factors. Systemic factors that affect fracture repair are well characterized in the literature [21, 25], and include endocrine, metabolic, and genetic factors and drug treatment. Local factors are appreciated as important in fracture repair, but are less well characterized. Local regulators of fracture repair could be secreted by both inflammatory and noninflammatory cells. Current investigations indicate that macrophages and other inflammatory cells at sites of injury in non-skeletal tissues secrete cytokines and growth factors that are critical regulators of healing. The presence of inflammatory cells in the fracture callus suggests that macrophages in the callus also secrete cytokines and growth factors to regulate the initial stages of fracture repair. The literature on growth factors effects on cells in vitro [5, 8, 26] contains many examples demonstrating growth factors regulation of musculoskeletal cell function, including stimulation of proliferation by chondrocytes, osteoblasts, and periosteal cells, initiation of chondrocyte differentiation and the expression of type II procollagen in the periosteum, and modulation of extracellular matrix synthesis by chondrocytes and osteoblasts. As similar cellular events occur in the fracture callus, these studies suggest that growth factors also act as regulators of cell differentiation and matrix synthesis in the later stages of fracture repair. Rapid progress in bone cellular and molecular biology has led to the identification of many signal molecules associated with the formation skeletal tissue, including members of transforming growth factor-β (TGF-β) superfamily [9, 27].

The role of TGF-β in the regulation of osteogenesis
Transforming growth factor-β belongs to large superfamily of related proteins that also includes bone morphogenetic proteins (BMPs). All members play important roles in regulating cell proliferation and differentiation and the production of extracellular matrix.
There are five isoforms of transforming growth factor-β (TGF-β1 to TGF-β5). Most cells synthesize and respond to TGF-β, but high levels are found in bone, platelets and cartilage. Transforming growth factor-β1 is the most abundant isoform at the protein level (for a recent comprehensive review see Chen et al. [28]). TGF-β1 is synthesized as a 390-amino acid protein (pre-pro-TGF-β1) consisting of three distinct pars: the signal peptide (SP; 29 amino acids), the latency-associated peptide (LAP; 249 amino acids), and the mature peptide (MP; 112 amino acids) (fig. 2, a). Two monomers dimerize by way of disulfide bridges between cysteine residues at positions 223 and 225 in the LAP and cysteine residue 356 in the mature peptide. The protein is cleaved by furin convertase at the dibasic arginine residue at position 278. This yields the LAP and the mature peptide. Noncovalent bonds between them prevent the premature activation of the mature peptide, forming the small latent complex or SLC — can become associated with a latent TGF-β binding protein to form the large latent complex. Because members of the TGF-β family are secreted as latent complex, they need to be activated to exhibit their biological activity. Activation of the latent complex initiates with its release from the extracellular matrix a process mediated by proteases (plasmin, leucocyte elastase, thrombin) that cleave the latent TGF-β binding protein at a protease-sensitive hinge region and target the cleaved complex to the cell surface. Once activated, TGF-β can interact its receptor to induce signaling (fig. 2, b). All members of the TGF-β superfamily signal through a dual receptor system of type I and type II transmembrane serine-threonine kinases [9, 29]. These receptors belong to a family of glycoproteins characterized by a cysteine-rich extraacellular region, a single transmembrane α-helix, and a cytoplasmic domain with a kinase domain (KD). In addition, the type I receptors share a highly conserved glycine- and serine-rich (GS) domain adjacent to the kinase domain, the GS domain. The type II receptors are characterized by their constitutively active kinase domain. In the absence of ligand, both type I and type II receptors are present as homodimers. Upon TGF-β1 binding to TGFβR II, TGFβRI I can be recruited into a heterotetrameric TGFβR II/TGF complex. Ligand-inducedimerization of the receptor complex is followed by transphosphorylation of the GS domain of TGFβR I by the constitutively phosphorylated TGFβR II kinase, resulting in activation of TGFβRI I. This transphosphorylation in the first step in the intracellular transmission of the signal through the superfamily signal effector (SMAD) family of molecules. Signaling through the downstream SMAD family of molecules is characteristic of the TGF-β superfamily member receptors (fig. 2, b). SMADs, a family of proteins, are important mediators in the TGF-β signaling cascade. SMAD2 and SMAD3 are bound to SARA (SMAD anchor for receptor activation) in the cytoplasm which presents SMAD2 and SMAD3 to the activated TGF-β receptor complex. TGF-β type I receptor then directly phosphorylates the carboxy terminal of SMAD2 and SMAD3, resulting in decreased affinity to SARA and heterotrimerization of SMAD2 and SMAD3 with SMAD4. This entire complex then translocates into the nucleus via the nucleoporins within the nuclear.
pore complex, and transcriptionally regulates multiple effector genes. The SMAD2/3/4 complex’s stay within the nucleus is transient, as it becomes dephosphorylated, and shuttled back out to the cytoplasm, where it becomes rephosphorylated to repeat its trip once again. In addition, several other lines of evidence point to the involvement of MAPK signaling pathways in transmitting TGF-ß signals from receptor to nucleus. In vitro kinase assays have demonstrated that TGF-ß can activate all three MAPK pathways, leading to ERK, c-Jun N-terminal kinase (c-JNK) and p38 MAPK and phosphorylation of members of the c-Jun, c-Fos, c-Myc and transcription factor families, which homo — and heterodimerize to form the activator protein (AP-1) (see reviewed in Ref. [5, 8]). Crosstalk between SMAD and MAPK pathways adds to the complexity of TGF-ß signaling. Signaling by TGF-ß family proteins regulates the differentiation and function of the bone-matrix-depositing osteoblasts and of the bone-matrix-resorbing osteoclasts, as well as the cross-talk between both cell types, which controls bone remodeling and homeostasis [29].

Roles of TGF-ß family in bone remodeling. Bone remodeling is a complex process involved a number of cellular functions directed toward the co-ordinated resorption and formation of new bone. Bone remodeling is regulated by systemic hormones and by local factors [24]. Hormones regulate the synthesis, activation, and effects of the local factors that have a direct action on cellular metabolism, and they modify the replication and differentiated function of cells of the osteoblast or osteoclast lineage.

Throughout life, bone tissue is continuously remodeled by the balanced processes of bone resorption and consecutive bone formation. Formation, deposition, and mineralization of bone tissue are executed by the osteoblasts that differentiate from mesenchymal precursor cells. The key transcription factor that drives the mesenchymal precursor cell toward the osteoblast lineage and controls bone formation is RUNX2 (Cbfa 1), which regulates the expression of all known marker genes expressed by the osteoblast [30]. Bone resorption by the osteoclasts involves demineralization of the inorganic matrix by acidification followed by enzymatic degradation of the organic matrix by cathepsin K and matrix metalloproteinases [31]. Osteoclasts are large, multinucleated cells of hematopoietic origin that differentiate from monocyte/macrophage precursor cells within the bone environment. The recognition that osteoclast differentiation requires the presence of marrow stromal cells or osteoblasts led to the discovery of the two osteoblast-derived factors essential and sufficient to promote osteoclastogenesis: macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B ligand (RANKL). Upon binding to their respective receptors on the osteoclast precursor cell surface (c-fms and RANK), two prominent transcription factor complexes, the NF-κB and NFATc-1 proteins, are activated, and signaling cascades essential for proper osteoclast differentiation, fusion, function, motility, and survival are initiated (Fig. 3). Osteoblasts also secrete a soluble inhibitor of osteoclast differentiation, osteoprotegerin (OPG), which acts as a «decoy» receptor for RANKL. OPG inhibits activation of the RANK receptor [30, 32]. A balance of these osteoclast promoting and inhibitory signals allows calibration and
coordination of bone deposition and bone resorption [32]. A pivotal role in the bone-remodeling process has been assigned to TGF-ß because it was proven to affect both bone resorption and formation. Bone formation by TGF-ß is promoted through chemotactic attraction of osteoblasts, enhancement of osteoblast proliferation and the early stages of differentiation with production of extracellular matrix proteins that compose the bone matrix, e.g. type I and II collagen, osteopontin, and osteonectin, as well as by the expression of the osteoblast differentiation markers, alkaline phosphatase (ALP) and, in a later stage osteocalcin. To better understand the complex roles of TGF-ß in bone metabolism, Karst M. et al. [33] examined the impact of a range of TGF-ß concentrations on osteoclast differentiation. In co-cultures of support cells and spleen or marrow osteoclast precursors, low TGF-ß concentrations stimulated while high concentrations inhibited differentiation. Authors investigated the influences of TGF-ß on macroage colony stimulating factor (M-CSF), receptor activator of NF-kB ligand (RANKL), and osteoprotergerin (OPG) expression and found a dose de inhibition of M-CSF and RANKL expression with a dramatic increase in OPG (fig. 3). From their findings, they conclude that osteoclast differentiation is stimulated at low TGF-ß concentrations because both the RANKL to OPG ratio and M-CSF levels are high. In contrast, at high TGF-ß concentrations, the RANKL to OPG ratio is repressed as TGF-ß suppresses RANKL expression and increases OPG expression by the osteoblast [33]. In combination with the dose-dependent inhibition by TGF-ß of M-CSF expression, this results in inhibition of osteoclast differentiation. Regarding the diversity of processes in which TGF-ß is involved, it is not surprising that this cytokine is of major importance both during embryogenesis and in maintaining bone homeostasis during life.

**Role transforming growth factor-ß in bone fracture healing.** TGF-ß is pleiot growth factor initially released by the degranulating platelets in the hematoma and the bone extracellular matrix at the fracture site [34]. In the initial stages of fracture repair, TGF-ß can be immunolocalized to the region of the hard callus where it defines the region of periosteal proliferation and intramembranous bone formation. Evidence suggests that TGF-ß is likely to be primarily involved in the stimulation of proliferation by the preosteoblasts in this region. In addition, the expression of TGF-ß is elevated during chondrogenesis and endochondral bone formation with an initial peak in mRNA levels detected around day 6 post fracture followed by a nadir at day 10. TGF-ß expression peaks again by day 14 and remains elevated until week 4. The nadir of TGF-ß expression correlates with the peak in type II collagen expression, and the subsequent peak temporally coincides with chondrocyte hypertrophy [7]. TGF-ß is primarily thought to be a stimulator of undifferentiated mesenchymal cell and chondrocyte proliferation and extracellular matrix production during chondrogenesis and endochondral bone formation. TGF-ß may also be involved in the normal coupling of bone formation with resorption [34]. The role of endogenous TGF-ß in normal fracture repair is inherently difficult to resolve. However, the importance of TGF-ß to this process is implied by the ability of exogenous TGF-ß to stimulate fracture repair in several models. The ability of TGF-ß to stimulate long bone healing was first demonstrated in midtibial osteotomies in rabbits treated with a compression plate. Continuous infusion of the osteotomy size with high doses of TGF-ß (1–10 μg/day) for 6 weeks resulted in a dose-dependent increase in callus volume and increased mechanical strength compared with untreated osteotomies. In a rat tibial fracture study, TGF-ß (4–40 ng) was injected around the fracture site every 2 days during a 40-day healing period. TGF-ß dose dependently increased the cross-sectional area of the callus, and mechanical testing demonstrated a higher ultimate load in fractures treated with the high dose of TGF-ß [35]. The results of these studies suggest that the ability of TGF-ß to stimulate fracture repair may require persistent dosing or very high concentrations.

**Bone morphogenetic proteins (BMPs).** The BMPs are a subfamily of the TGF-ß superfamily of polypeptides. The BMPs play critical roles in regulating cell growth, differentiation, and apoptosis in a variety of cells during development, including osteoblasts and chondrocytes. Compared with TGF-ß, BMPs have more selective effects on bone and also have shown more promising results in animal models of fracture healing. BMP signal transduction occurs by a mechanism similar to the other members of the TGF-ß superfamily (fig. 2). BMP ligand can associate with several serine-threonine kinase receptors, including BMP receptor type II, receptor type I A, and receptor type IB as well as the related activin receptors (ActR-II, ActR-I) [28]. As with TGF-ß, the BMP ligand binds to the type II receptor, and this receptor occupancy leads to association of the complex with an appropriate type I receptor forming an active receptor-ligand complex. This interaction can be blocked by the antagonists of BMPs, noggin and chordin, which can bind and block BMP activity by preventing receptor binding [36, 37]. This antagonist function of noggin and chordin has been specifically demonstrated in osteoblastic cells. The expression of the BMP receptors is dramatically increased in osteogenic cells of the periosteum near the
factors, including TGF-ß1 and BMPs, are released begins immediately following injury, when growth development of the fracture callus. Fracture repair tory roles for TGF-ß1 and BMPs in the initiation and steps in this cascade. Recent studies suggest regul

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is interesting to note that while mature osteoblasts and chondrocytes only levels of expression. The current view of the role of BMPs in fracture repair [39] is that these molecules are primarily activators of differentiation in osteoprogenitor and mesenchymal cells destined to become osteoblasts and chondrocytes. This activation by BMPs, specifically BMP-2, is inhibited by the molecules noggin and chordin which have been demonstrated to block BMP-2 interaction with its receptor [40]. As these primitive cells mature, BMP expression is dramatically reduced. BMP expression emerges transiently in chondrocytes and osteoblasts during their respective periods of matrix formation, and returns to low levels during callus remodeling. It is interesting to note that while mature osteoblasts and chondrocytes do not express significant levels of BMPs in normal bone, they both have greatly increased BMP expression later in fracture repair.

Concluding remarks

Fractured bones heal by a cascade of cellular events in which mesenchymal cells respond to unknown regulators by proliferating, differentiating, and synthesizing extracellular matrix. Current concepts suggest that growth factors may regulate different steps in this cascade. Recent studies suggest regulatory roles for TGF-ß1 and BMPs in the initiation and the development of the fracture callus. Fracture repair begins immediately following injury, when growth factors, including TGF-ß1 and BMPs, are released into the fracture hematoma by platelets and inflammatory cells. TGF-ß1 and BMPs are synthesized by osteoblasts and chondrocytes throughout the healing process. TGF-ß1 and BMPs appear to have an influence on the initiation of fracture repair and the forming of cartilage in intramembranous bone in the initiation of callus formation. These studies suggest that TGF-ß1 and BMPs are central regulators of cellular proliferation, differentiation, and extracellular matrix synthesis during fracture repair. The explosion of knowledge and the understanding of the role of TGF-ß1 and BMPs, their mechanisms of action and molecular signaling pathways, which have been reviewed in this article, suggest the potential for many novel therapeutic targets, not only for applying growth factors but also for the potential use of growth factor inhibitors or agents that target specific parts of the intracellular signaling pathways. There remains an enormous challenge to convert some of the knowledge from basic studies of bone cell physiology to therapeutically useful techniques for the future. We are optimistic that such novel approaches may result in real qualitative improvement in clinical outcomes over currently available techniques.

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КЛЕТОЧНАЯ И МОЛЕКУЛЯРНАЯ БИОЛОГИЯ СРАЩЕНИЯ ПЕРЕЛОМОВ КОСТЕЙ: РОЛЬ ТРАНСФОРМИРУЮЩЕГО ФАКТОРА РОСТА-SS1 В АКТИВИЗАЦИИ РЕПАРАТИВНОГО ОСТЕОГЕНЕЗА (ОБЗОР ЛИТЕРАТУРЫ)

С. Сагаловски, М. Шенерт

Ортопедическое отделение, Медицинская клиника, Бад-Лауэнзик, Германия

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