Invited Review

Molecular signaling in bone fracture healing and distraction osteogenesis

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Summary. The process of fracture healing has been described in detail in many histological studies. Recent work has focused on the mechanisms by which growth and differentiation factors regulate the fracture healing process. Rapid progress in skeletal cellular and molecular biology has led to the identification of many signaling molecules associated with the formation of skeletal tissues, including members of the transforming growth factor-β (TGF-β) superfamily and the insulin-like growth factor (IGF) family. Increasing evidence indicates that they are critical regulators of cellular proliferation, differentiation, extracellular matrix biosynthesis and mineralization. Limb lengthening procedure (distraction osteogenesis) is a relevant model to investigate the in vivo correlation between mechanical stimulation and biological responses as the callus is stretched by a proper rate and rhythm of mechanical strain. This model also provides additional insights into the molecular and cellular events during bone fracture repair. TGF-β1 was significantly increased in both the distracted callus and the fracture callus. The increased level of TGF-β1, together with a low concentration of calcium and an enhanced level of collagen synthesis, was maintained in the distracted callus as long as mechanical strain was applied. Less mineralization is also associated with a low level of osteocalcin production. These observations provide further insights into the molecular basis for the cellular events during distraction osteogenesis.

Key words: Growth factors, fracture healing, Distraction osteogenesis, Mechanical strain

Introduction

It is common knowledge that bone tissue has a substantial capacity for regeneration and repair in response to injury. Bone healing is in some way very similar to the healing of soft tissues as they both require the cellular regulation of chemotaxis, proliferation, differentiation, extracellular matrix synthesis and formation of new tissue at the site of injury. In contrast to the frequent scar formation during soft tissue healing, bone heals by regeneration of the normal osseous anatomy without formation of scar tissue. The cellular events associated with the repair of bone injury involve, in addition to proliferation of progenitor cells and the influx of inflammatory cells also found in soft tissue wounds, the formation of bone by means of intramembranous and endochondral ossification. Cells participating in this process include platelets, monocytes, mesenchymal cells, fibroblasts, endothelial cells, chondrocytes, osteoblasts, and osteoclasts (Erlebacher et al., 1995). Among these types of cells, osteoblasts play a critical role in this process, because they are responsible for the synthesis and mineralization of the bone matrix (Rodan and Noda, 1991; Aubin et al., 1992; Gehron Robey et al., 1992; Rodan, 1992).

Limb lengthening procedures (distraction osteogenesis) consist mainly of an osteotomy of the shortened bone and a subsequent daily distraction of the newly formed callus leading to new bone formation. The clinical importance of the treatment is to fill large bone defects caused by infection, accidental injury, tumor resection, or congenital deformation, and to retain the original bone length. With adequate blood supply, rigid fixation, and proper rate and rhythm of distraction, a large amount of new bone rapidly develops within the distraction gap. This biological phenomenon appears to contradict the dogma that a fracture subjected to stretching forces may become a risk for a nonunion. However, a large number of studies have shown that bone formation activity is markedly increased during distraction osteogenesis (Monticelli et al., 1981; Alho et al., 1982; Paley, 1988; Ilizarov, 1989; Aronson, 1991; Peltonen et al., 1992). This increased activity has been attributed to the “tensioning” stimulatory effect on blood flow and bone forming cells, which results in rapid regeneration, bone formation and consolidation. To further understand this phenomenon, it is of importance, using modern tools of molecular biology, to analyze the
molecular mechanisms associated with this process.

Bone fracture healing is believed to be regulated by systemic and local factors. Recent studies have demonstrated that growth factors are central regulators of cellular proliferation, differentiation, and extracellular matrix biosynthesis and mineralization during this process. This paper is a brief overview of recent progress from studies which demonstrated the expression of local growth factors associated with cellular events in both in vitro and in vivo models. Although bone forming cells synthesize and release many growth factors and cytokines such as platelet-derived growth factor (PDGF) and fibroblast growth factors (FGFs), we will focus on transforming growth factor beta (TGF-β), bone morphogenetic proteins (BMPs) and insulin-like growth factors (IGFs) because there is growing evidence that these growth factors function in the regulation of bone formation and their actions on bone formation have been extensively investigated.

Growth factors are present during fracture healing

The bone fracture healing process has been histologically divided into four distinct stages: (a) hematoma and immediate injury response; (b) primary intramembranous bone formation; (c) chondrogenesis; and (d) endochondral ossification. Each stage is characterized by specific cellular and matrix markers (Bolander, 1992). Cellular events involved in this process include chemotaxis of mesenchymal precursors and proliferation of committed bone-forming cells occurring in the early stage of differentiation at the beginning of the fracture healing process (Iwaki et al., 1997). Cells subsequently undergo differentiation and synthesize distinct extracellular matrix, leading to intramembranous and endochondral ossification with formation and mineralization of a fracture callus. Finally, most cells are removed from the fracture site via apoptosis (Landry et al., 1997). Cell populations associated with these events mainly arise from mesenchymal precursors present in the bone marrow, including the endosteum, and the periosteum. These precursor cells can undergo osteogenic, chondrogenic, myogenic, tendogenic, ligamentagenic, and adipogenic differentiation forming a variety of mesenchymal tissues, including cartilage and bone (Bruder et al., 1994). In case of a fracture, the precursor cells enter a lineage following their commitment to particular chondrogenic and osteogenic pathways.

The above mentioned cellular events are induced and controlled by specific molecular signals, including TGF-βs, BMPs and IGFs. Growth factor regulation may function via one or all of three pathways: endocrine, paracrine, and autocrine (Joyce et al., 1991). In addition, specific receptors and cytoplasmic signal transducers, e.g. recently discovered members of the SMAD family, are crucially involved (Heldin et al., 1997; Baker and Harland, 1997). Recent studies demonstrated that these growth factors, as well as their receptors and signal transducer proteins, are present in the fracture callus.
with distinct expression patterns during different stages of the fracture healing process as reviewed in Table 1.

**TGF-β**

A number of in vitro studies have shown that TGF-β has a variety of effects on osteoblast-like cells, depending on the source of cells, culture conditions and other parameters. Judging from these in vitro studies, however, the primary function of TGF-β appears to be the stimulation of cell proliferation and matrix biosynthesis (Boyan et al., 1994; Centrella et al., 1994; Harris et al., 1994; Ballock et al., 1997). This is supported by a recent study on the temporal expression pattern of TGF-β in a fetal rat calvarial-derived osteoblast model (Owen et al., 1990). In this model, TGF-β and collagen type I were co-expressed during the proliferation period of osteoblast growth, then gradually down-regulated with collagen mRNA being maintained at a low level during osteoblast maturation. Several lines of evidence indicated that TGF-β has an inhibitory effect on osteoblast differentiation (Talley Ronsholdt et al., 1995; Cheifetz et al., 1996). On the other hand, TGF-β1 has been shown to stimulate chondrogenic differentiation in periosteal mesenchymal cell cultures (Izumi et al., 1992; Iwasaki et al., 1993, 1995; Miura et al., 1994) and osteoblast-like cell cultures (Basic et al., 1996). In the in vivo studies, both TGF-β1 and -β2 have been reported to stimulate bone formation by inducing differentiation of periosteal mesenchymal cells into chondroblasts and osteoblasts (Noda and Camilliere, 1989; Joyce et al., 1990b). Noteworthy, TGF-βs dose-dependently increased the ratio of cartilage formation to intramembranous bone formation and the endochondral ossification just occurred after discontinuing daily injection of TGF-βs (Joyce et al., 1990b). Interestingly, TGF-β1 with a collagenous carrier matrix was reported to promote endochondral bone formation in extraskeletal sites in adult baboons (Ripamonti et al., 1997).

Using Northern analysis, in situ hybridization and immunohistochemistry, it is possible to investigate the expression and presence of individual growth factors within the fracture site during fracture repair. Joyce et al. (1990a) have demonstrated that extracellular TGF-β1 was immunohistochemically localized in the hematoma as early as 24 hr post fracture and mRNA for TGF-β1 was up-regulated during chondrogenesis and endochondral ossification. Andrew et al. (1993a) have shown that TGF-β1 mRNA was detected by in situ hybridization in proliferating mesenchymal cells in granulation tissue at the early stage of fracture repair, and in proliferating plump polygonal osteoblasts and small chondrocytes during the matrix formation stage. Consistently, Si et al. (1997) have also reported that TGF-β1 mRNA was higher in chondrocytes and active differentiated osteoblasts during the stages of chondrogenesis and endochondral ossification in the rabbit mandible fracture healing model. Recently, Lammens et al. (1998) have shown that in a dog tibia fracture healing model, the level of TGF-β1 protein in callus extracts was increased within the first month, peaked by 5 weeks after fracture, and then gradually decreased down to a basal level by 13 weeks post fracture. Meanwhile, an approximate similar synthesis pattern of collagen was documented in the same model.

TGF-β receptors have been identified and characterized in osteoblasts (Kells et al., 1992; Takeuchi et al., 1995). Receptors (ActRI, II, IIB) for activin, a TGF-β superfamily member, have been reported to be expressed by osteoblasts during fracture healing (Shuto et al., 1997). The Smad 2, 3 and 4 are intracellular signal transducer proteins in the TGF-β/activin signaling transductive pathway (Heldin et al., 1997). Recently, the expression of Smad 2, 3, and 4 was documented in a rat fracture healing model (Matsui et al., 1998). Analysis of the Smad expression indicates that TGF-β mediated signaling is restricted to a region of the chondroblast proliferation and differentiation during the stages of chondrogenesis and endochondral ossification.

These studies revealed that TGF-β arrives at the fracture site by the blood stream, most likely released by platelets, and is subsequently synthesized by bone forming cells within the callus throughout the fracture repair process. It may also be speculated that TGF-β, along with other growth factors such as PDGF and FGFs, plays an important role to unlock cellular proliferation in mesenchymal cells, and together with other factors such as BMPs, induces differentiation of periosteal mesenchymal cells into chondroblasts and osteoblasts at the early stage of fracture repair. During the stages of chondrogenesis and endochondral ossification, TGF-β stimulates the proliferation and synthetic activity of chondrocytes and osteoblasts, increasing the fracture callus size (Andrew et al., 1993a). However, TGF-β signaling appears not to be required during the later phases of mineralization and remodeling (Le et al., 1998).

**BMPs**

The BMPs are a group of related proteins originally isolated and characterized from bovine bone matrix by their ability to ectopically induce endochondral bone formation, and form a unique subfamily within the TGF-β superfamily. By molecular cloning, six related members of this family were originally identified and are termed BMP-2 through BMP-7 (also called osteogenic protein 1, OP-1). To date, the BMP-family has been continuously expanding (Reddi, 1997). Since it was first described by Urist (1965), the regulatory effects of BMPs on bone and cartilage formation have been extensively investigated in both in vitro and in vivo studies, using heterotopic/orthotopic bone formation, fracture healing, and embryogenesis. Findings from developmental biology and cell culture studies suggest that BMPs are critically involved in the osteoblastic and chondroblastic differentiation. BMPs were reported to induce cartilage and bone formation when injected.
subcutaneously, intramuscularly, or periosteally into adult rats or other species (Reddi, 1997). BMPs are expressed in mouse embryos at sites of cartilage and bone formation (Hogan, 1996). BMPs were also shown to suppress myogenic expression and to induce chondrogenic or osteogenic expression both in multipotent and committed cell lines. Furthermore, the inductive effect of BMPs on osteoblast differentiation is strongly supported by a recent study which demonstrated that BMP-7 induced expression of Osf2/Cbfa1, a transcription factor associated with early osteoblast differentiation, in cells where it is not normally expressed, prior to the expression of any other osteoblast marker genes (Ducy et al., 1997).

The temporal and spatial expression patterns of some BMPs and their receptors, BMP type I and type II receptors (BMPR I and II), have been defined during fracture healing process in several in vivo models. Nakase et al. (1994) reported that mRNA for BMP-4 was induced very rapidly in less differentiated osteoprogenitors of the proliferating periosteum near the fracture site in the early stage of mouse fracture repair before new cartilage and bone formation, and decreased to an undetectable level on day 5 after fracture. The BMP-4-positive cells did not express bone Gla protein mRNA, which is a marker of more mature osteoblasts. Recently, Si et al. (1997) have demonstrated that BMP-2 mRNA was increased in undifferentiated mesenchymal cells, differentiating osteoblasts and chondroblasts at the stage of intramembranous bone formation and early chondrogenesis. Consistently, Onishi et al. (1998) have also observed that BMP-2/4 and OP-1 were detected in various types of chondrocytes; strongly in fibroblast-like spindle cells and proliferating chondrocytes. In the meantime, BMPR I and II receptors are expressed by various types of cells within the healing callus throughout the fracture repair process (Ishidou et al., 1995). These findings, taken together with the abundance of in vitro and in vivo data on chondrogenic and osteogenic activities, strongly suggest that BMP signaling is associated with the differentiation of mesenchymal cells into the chondroblastic and osteoblastic lineage during fracture healing.

**IGFs**

IGFs have been reported to have a mitogenic activity for cells of the osteoblastic lineage and to stimulate osteoblast differentiated functions such as type I collagen and osteocalcin synthesis in vitro (Canalis et al., 1993; Dequeker et al., 1993; Mundy, 1993; Rosen et al., 1994; Mohan et al., 1995). Significant expression levels of IGFs were found in cells of developing periosteum, growth plate, and ectopic bone formation (Edwall et al., 1992; Prisell et al., 1993; Sandberg et al., 1993). These observations suggest that IGFs are involved in cartilage and bone formation and may play a role in fracture healing. In the past few years, several in vivo studies have shown that IGFs are expressed in fracture healing callus by immunohistochemistry and in situ hybridization. Bourque et al. (1993) have reported that IGF-I protein was present in young chondroblasts at the edge of the cartilage during the chondrogenesis stage of the fracture healing. Edwall et al. (1992) have shown that the level of IGF-I mRNA in callus peaked on day 8 post fracture. IGF-I immunostaining was found in cartilaginous cells, osteoblasts, and myocytes in the fracture site, respectively 6 and 8 days after fracture. Andrew et al. (1993b) also reported that mRNA expression for both IGF-I and II was detected in osteoblasts and non-hypertrophic chondrocytes at the stage of matrix formation and remodeling. Recently, Lammens et al. (1998) have demonstrated that IGF-I protein in callus extracts was increased at 13 weeks after fracture compared to normal bone. Interestingly, IGFs have been reported to increase osteoclast formation from mouse osteoclast precursors (Mochizuki et al., 1992; Hill et al., 1995). Andrew et al. (1993b) have also observed that IGF-II mRNA was detected in some osteoclasts in a fracture healing model. IGF-II-expressing osteoclasts were adjacent to osteoblasts that also expressed IGF-II, whereas most other osteoblasts at the stage of bone remodeling were negative for IGF-II. These findings suggest that IGFs are not only involved in proliferation and differentiation of osteoblastic and chondroblastic lineages, contributing to bone formation, but may also modulate osteoclast function, leading to bone remodeling during fracture repair.

**Growth factors are present during distraction osteogenesis**

Distraction osteogenesis has to be considered as a particular form of fracture healing, as it differs from callus formation or osteonal (primary) bone healing. After a percutaneous osteotomy, the newly forming bone is subjected to a mechanical longitudinal strain, allowing for the formation of large amounts of callus. This callus appears to result mainly from intramembranous ossification (Lavrishcheva and Mikhailova, 1985; Aronsen and Harrison, 1987; Jazrawi et al., 1998; Delloye et al., 1990). Distraction osteogenesis can be dynamically divided into three stages: a) latent stage; b) distraction stage; and c) consolidation stage (Fig. 1). In the latent stage (normally one week after osteotomy), similar to fracture healing, the hematoma is replaced by granulation tissue and intramembranous bone formation occurs flanking the granulation tissue under the periosteum. During the distraction stage, with gradual and continuous distraction, the maturation of newly forming bone in the distraction gap appears different, some areas are more calcified while the newly regenerated tissue elsewhere is less dense with more cellular and vascular components (Costantino et al., 1990). Radiological examination has also shown that there are high and low radiodensity zones in the
distraction gap. In combination with radiographic data, the distracted callus can be histologically divided into the growth zone and the mineralization zone. The growth zone is composed predominantly of longitudinal fibres and fibroblast-like cells (Kojimoto et al., 1988; Yasui et al., 1997). The majority of proliferating cells are present in the edge of the growth zone during distraction (Kojimoto et al., 1988; Aronson et al., 1997; Li et al., 1997). In the consolidation stage, growth zone is calcified, and in the meantime, newly formed bone is gradually remodeled into lamellar bone.

The mineralization was reported to begin from both sides adjacent to the original cortices in the callus during the distraction period (Delloye et al., 1990; Lammens et al., 1997). In the past several years, numerous in vitro studies have shown that proliferation and differentiation of bone forming cells (osteoblasts and chondrocytes) are subjected to regulation by mechanical strain (Meikle et al., 1979; Somjen et al., 1980; Hasegawa et al., 1985; Buckley et al., 1988; Burger et al., 1992; Harter et al., 1995; Stanford et al., 1995). By compiling these data and correlating the magnitudes of strain, it appears that at high levels of strain, cells increase their proliferative activity. At lower levels of strain, the response of cells indicates a more differentiated state. As the magnitude of strain is heterogeneous across the distraction gap, osteoblasts that experience the greatest strain increase their proliferative activity and, on the other hand, osteoblasts that experience less strain respond with differentiation, which results in mineralization.

Although increasing evidence indicates that mechanical strain induces a large amount of newly forming bone in the distraction gap, there is no significant difference in the bone formation rate between distraction osteogenesis and fracture healing (Welch et al., 1998). In distraction osteogenesis, therefore, bone forms just as rapidly as it does during fracture repair, but as long as mechanical strain is applied. Thus, a number of questions concerning distraction osteogenesis have arisen: a) why can mechanical strain stimulate and maintain the process of osseous regeneration? and b) does mechanical strain induce any specific growth factor that is involved in the distraction cellular events? Over the past few years, it has been shown by in vitro and in vivo studies that mechanical strain induces the expression of some growth factors such as TGF-βs, BMPs and IGF-1, which showed different expression patterns when compared to normal bone fracture repair (Table 2). Holbein et al. (1995) reported that cyclic stretching significantly increased the production of active TGF-β and cellular proliferation in primary human osteoblast culture. The level of TGF-β was increased in the sera from patients who had distraction between the third and fourth weeks during distraction. Lammens et al. (1998) have recently demonstrated that, in a canine tibia distraction model, the level of TGF-β1 protein was increased in distracted callus as long as continuous traction was applied, whereas TGF-β1 in osteotomized callus was first increased and gradually decreased to a basal level afterwards. Consistently, levels of TGF-β1 in sera from dogs with a tibia distraction were increased immediately after application of distraction and then maintained at a high level during the whole experimental period. In addition, a constantly increased level of collagen synthesis and low concentration of calcium were observed in distracted callus during the distraction period. Recently, Tay et al. (1998) reported that mRNA for TGF-β2 was detected by in situ hybridization in the growth zone of the distracted callus during the distraction period. These findings suggest that the mechanical strain stimulates and maintains callus formation by inducing TGF-β production, possibly preventing differentiation and delaying mineralization.

Osteocalcin is a phenotypic marker of osteoblasts and is expressed during the mineralization stage of osteoblasts. The relationship between proliferation and phenotypic expression has been well characterized in the calvarial osteoblast differentiation model (Lian et al., 1991). In that model, subconfluent calvarial-derived

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**Table 2. Expression of morphogens, collagenous and non-collagenous proteins in distraction osteogenesis.**

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Latent</th>
<th>Distraction</th>
<th>Consolidation</th>
</tr>
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<tbody>
<tr>
<td>TGF-β1</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>BMP-2/4</td>
<td>▲</td>
<td>▲</td>
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<tr>
<td>IGF-1</td>
<td>ND</td>
<td>NC</td>
<td>▲</td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>ND</td>
<td>▼</td>
<td>▼</td>
</tr>
<tr>
<td>Collagen</td>
<td>ND</td>
<td>▲</td>
<td>▲</td>
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</tbody>
</table>

▲: increased compared to normal bone; ▲▲: dramatically increased compared to normal bone; ▼: decreased compared to normal bone; ND: not determined; NC: no change compared to normal bone.

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**Fig. 1. Models for distraction osteogenesis.**

GT: Granulation tissue. IB: Intramembranous ossification.

GZ: Growth zone. MZ: Mineralization zone.

GZ: Growth zone has been calcified.
A hypothesis and reminiscence

Findings from histological examination suggest that mechanical strain stimulates and maintains the process of osseous regeneration most probably by maintaining the proliferative state of osteoprogenitors in the growth zone. Thus, the biological function of the growth zone in distraction osteogenesis appears in some way very similar to the "progress zone" (PZ) in vertebrate limb development. Indeed, the maintenance of the PZ is critical for outgrowth of limb bud (Johnson and Tabin, 1997). FGFs, as well as other related molecular signals such as WNTs, have been demonstrated to play important roles in this context (Johnson and Tabin, 1997; Kengaku et al., 1998). Moreover, chondrogenesis was reported to be inhibited by FGF-2 (Kato and Iwamoto, 1990), Wnt-1 (Rudnicki and Brown, 1997), or mechanical strain (Jazrawi et al., 1998). These observations may provide a clue that these molecular signals might be additional players in distraction osteogenesis. Recently, Indian hedgehog (Ihh) and parathyroid hormone-related protein (PTHrP) have been shown to be involved in endochondral bone formation in both embryonic development and fracture healing (Vortkamp et al., 1998). These data further corroborate the hypothesis that bone formation in these processes, including distraction osteogenesis, is most likely controlled by similar molecular mechanisms.

Conclusion

Fracture healing is a complex tissue regeneration and repair process which involves chemotaxis of mesenchymal cells, proliferation and differentiation of committed bone-forming cells, extracellular matrix synthesis and callus formation, maturation and remodeling. Current data suggest that these cellular events are precisely controlled and regulated by specific morphogens, while such molecular signals are also implicated in bone formation during embryonic development. The secreted signals exert their functions via a cascade of molecular events which consist of biosynthesis, secretion, activation, transportation, signal transduction and gene expression. Indeed, these molecular events are believed to be influenced by many factors, including blood supply, mechanical stimuli, extracellular and intracellular microenvironment, etc.

Recent studies have demonstrated that a number of morphogens are involved in fracture repair, including TGF-β, BMPs and IGFs. Additional data are required to identify the specific functions of these morphogens in the bone healing process. TGF-β most probably functions as a regulator to stimulate proliferation of bone forming cells and extracellular matrix synthesis. BMPs may stimulate differentiation of mesenchymal cells into...
osteoblastic and chondroblastic lineage, leading to intramembranous bone formation and endochondral ossification in fracture callus. IGFs seem not only to regulate proliferation and differentiation of bone forming cells, contributing to bone formation, but also to regulate osteoclast function, participating in bone remodeling. The mechanical strain can stimulate and maintain the process of osseous regeneration by inducing specific morphogen expression, synthesis and processing.

References


Morphogens in fracture healing and distraction osteogenesis


Accepted September 19, 1998