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■ REVIEW ■

Angiogenesis and Mineralization During Distraction Osteogenesis

Distraction osteogenesis is currently a standard method of bone lengthening. It is a viable method for the treatment of short extremities as well as extensive bone defects, because large amounts of bone can be regenerated in the distraction gap. Mechanical stimulation by distraction induces biological responses of skeletal regeneration that is accomplished by a cascade of biologic processes that may include differentiation of pluripotential tissue, angiogenesis, mineralization, and remodeling. There are complex interactions between bone-forming osteoblasts and other cells present within the bone microenvironment, particularly vascular endothelial cells that may be pivotal members of a complex interactive communication network in bone. Regenerate bone forms by three modes of ossification, which include intramembranous, enchondral, and transchondroid ossifications, although intramembraneous bone formation is the predominant mechanism of ossification. In this review we discussed the coupling between angiogenesis and mineralization, the biological and mechanical factors affecting them, the cellular and molecular events occurring during distraction osteogenesis, and the emerging modalities to accelerate regenerate bone healing and remodeling.

Key Words : *Osteogenesis, Distraction; Neovascularization, Physiologic; Bone Mineralization*

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INTRODUCTION

Distraction osteogenesis, currently a standard method of bone lengthening, is based upon the "tension-stress principle", as proposed by G.A. Ilizarov (1-3). The essence of this technique is the gradual distraction of a fracture callus after low-energy "corticotomy" of the long bone with careful preservation of the soft tissue envelope surrounding the bone. The indications of distraction osteogenesis for reconstructive operation have been rapidly widened in the fields of orthopedic, craniofacial, and maxillary surgery, since the introduction of this technique to the western world in early 1980s.

Extensive animal experiments have markedly expanded the understanding of the histological, radiographic, biochemical, vascular, and biomechanical properties, as well as the soft tissue effects, of distraction osteogenesis (4-18). Distraction osteogenesis shares many features of embryonic growth, fetal growth, and neonatal limb development (3), as well as normal fracture gap healing (19). However, the exact cellular and molecular mechanisms of osseous and non-osseous regeneration are still not well understood. Ample evidence has emphasized the contribution of both periosteum and local neovascularity on bone formation during distraction (18, 20). Ilizarov (1-3) claimed that the shape and size of the bone are influenced by the amount of load applied on the bone and its blood supply. When accompanied by a corresponding increase in the blood supply, an increase in the load on a bone would lead to an increase in bone size. Recent molecular investigations indicate that the growth factor cascade is likely to play an important role in distraction. Danis (21) hypothesized that distraction osteogenesis of long bone relies on two local factors: (a) mechanical stret ching multiplicates the fibroblastic population of undifferentiated mesenchymal cells; (b) hypoxia, by vessel elongation and cellular compaction, induces osteogenic stress protein metabolism. Progressive return to aerobic conditions by neoangiogenesis assures the permanency of the new osseous structures.

The purposes of this review are to discuss the relationship between angiogenesis and mineralization, the biological and mechanical factors affecting them, the cellular and molecular events occurring during distraction osteogenesis, and the emerging modalities to accelerate regenerate bone healing and remodeling.

HISTOLOGICAL, RADIOGRAPHIC, AND VASCULAR FEATURES OF DISTRACTION OSTEOGENESIS

Histological variations have been reported in the distraction zone. However, most histological investigations of the Ilizarov method have confirmed that in contrast to fracture healing, the mode of bone formation in distraction osteogenesis is primarily intramembranous ossification (1-3, 5, 8,

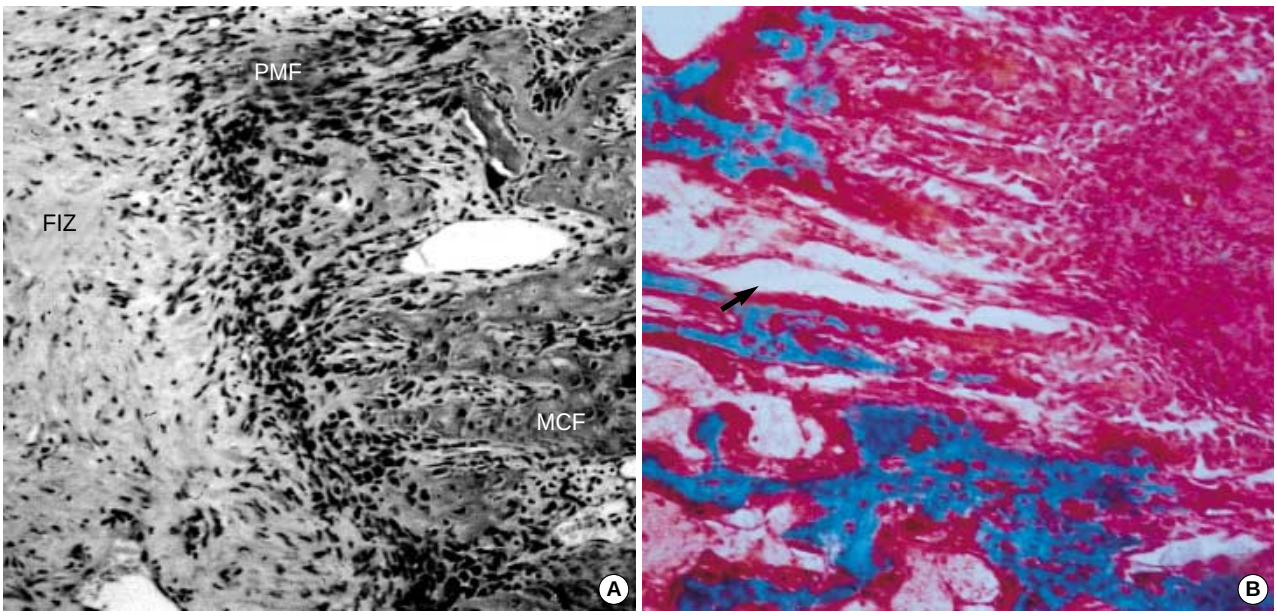

Fig. 1. A typical histological zonal pattern is seen in a model of rat tibial lengthening by distraction osteogenesis (0.5 mm/day in two increments). (A) The highest proliferating cell density is observed in the primary matrix front (PMF). Longitudinal columns of new bone reach maximum diameters of 150-200 μm. Fibrous interzone (FIZ) stands for fibrous interzone, PMF for primary mineralization or matrix front, and micro column formation (MCF) for microcolumn formation (H&E, $\times$100). (B) Newly formed vascular sinuses (150-250 μm in diameter) and vessels (arrow) are oriented in the same direction as the microcolumns of new bone which appear to be the sites from which bone formation is initiated within the distraction gap (H&E, $\times$100).

10, 11, 18, 22-26) occurring in uniform zones. A central zone, called the fibrous interzone (FIZ), comprised of type-I collagen (26), bridges adjacent zones of vascular ingrowth, where proliferating and differentiating osteoblasts deposit osteoid along the collagen bundles. The cells in the interzone show high levels of alkaline phosphatase, pyruvic acid, lactic acid, and enzymes for oxidation-reduction (3). The newly formed vascular sinuses (150-250 μm in diameter) appear to be the sites from which bone formation was initiated within the distraction gap. As the distraction gap increases, the longitudinal columns of bone that had crystallized longitudinally along the oriented collagen bundles increase in length and in diameter, while the FIZ remains about 4 mm long. Histologically, the bone columns, called zone microcolumn formation (MCF) by Aronson et al. (10), resemble stalagmites and stalactites, in microradiography and scanning electron microscopy (EM) images, and project from each corticotomy surface toward the center. These cones reach maximum diameters of 150-200 μm at the corticotomy surfaces (Fig. 1). When distraction is stopped, the gap begins to consolidate. The columns of bone produced from the local host surfaces are eventually interconnected, and quickly remodel to the equivalent macro and microstructure (5, 7). The enhanced bone formation and remodeling appear to result more from increased recruitment and activation of bone forming and resorbing cells rather than from an increased level of individual cellular activity. Several authors (14, 16, 17, 27, 28) confirmed sustained cell proliferation during the distraction period by immunohistochemical staining with bromodeoxyuridine or proliferating cell nuclear antigen (PCNA), and by ^{3}H-thymidine. The highest proliferating cell density is observed in the zone between the FIZ and MCF. Aronson et al. (10) called this transitional zone the primary matrix or mineralization front (PMF). We observed in a transmission EM study of the rat tibial lengthening model (16-18, 27) that the cells in the distraction site are metabolically very active, showing hypertrophied mitochondria, endoplasmic reticulum, nucleoli, and Golgi complex. We could verify that during the distraction period, preosteoblasts aligned among the elongated collagen fibers in the PMF and MCF along the direction of stretching. In the late distraction and early consolidation period, preosteoblasts differentiated into osteoblasts that were subsequently surrounded by mineralized matrix at the PMF, and eventually became osteocytes when the matrix was fully mineralized, encasing the cells (Fig. 2).

The progression of healing within the distraction gap from the central zone of collagenous growth to the more peripheral columns of mineralized bone results in a distinctive radiographic appearance (6, 10, 11, 29). During the distraction period three distinct zones are usually observed: a central radiolucent zone (interzone), a zone of increased bone density (zone of sclerosis), and a zone with low density (zone of remodelling) (29) (Fig. 3). The time sequence of radiographic bone formation at the metaphyseal site has been measured experimentally. Aronson (5, 6, 10) reported that the rate of linear bone formation ranged from 200 to 400 μm/day in the experimental models. This is 4 to 8 times faster than the fastest physeal

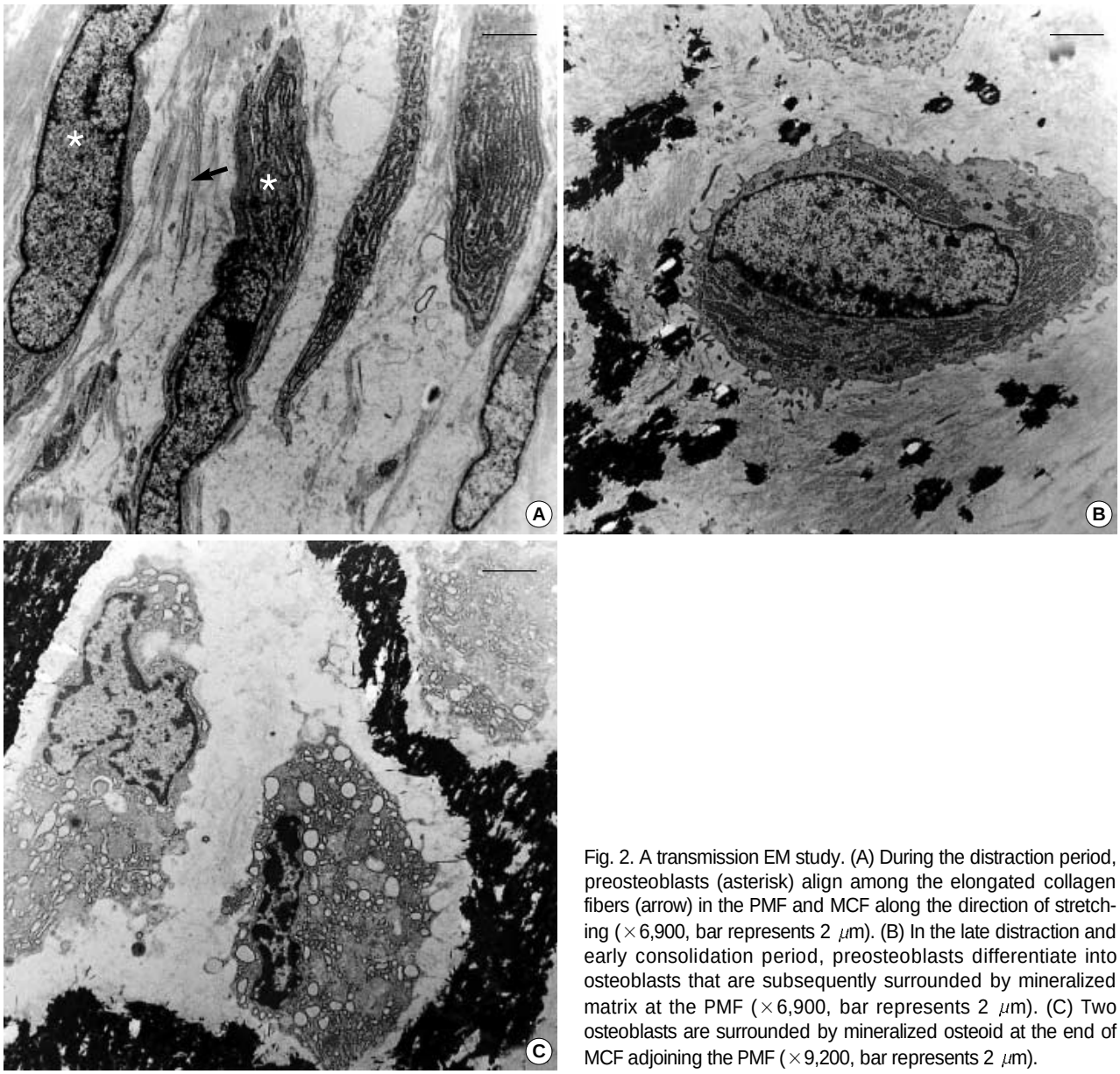

Fig. 2. A transmission EM study. (A) During the distraction period, preosteoblasts (asterisk) align among the elongated collagen fibers (arrow) in the PMF and MCF along the direction of stretching (×6,900, bar represents 2 μm). (B) In the late distraction and early consolidation period, preosteoblasts differentiate into osteoblasts that are subsequently surrounded by mineralized matrix at the PMF (×6,900, bar represents 2 μm). (C) Two osteoblasts are surrounded by mineralized osteoid at the end of MCF adjoining the PMF (×9,200, bar represents 2 μm).

growth in an adolescent (50 μm/day) and is equivalent to that occurring in the fetal femur. Maffulli et al. (30) reported, based on dual-energy radiography absorptiometry (DEXA), that mineralization of the regenerate after completion of the lengthening process reached levels significantly greater than at removal of the fixator, with an increase of greater than 50% of the prelengthening values, regardless of the underlying pathology. The final value of this increased bone mineral content (BMC) was not significantly different from that in the normal contralateral unoperated limb.

Mode of Ossification

Histologic and molecular events occurring during distraction osteogenesis share many features of normal fracture gap healing, particularly during the latency period. But, the speed of new bone formation in distraction osteogenesis is twice as fast (7). After 3 to 4 weeks of distraction, standardized radiographs of experimental specimens demonstrate a central radiolucent gap, although histological studies, quantitative computed tomography, and DEXA demonstrate, as early as the tenth day of distraction, deposition of new bone mineral in this gap (5, 6, 10, 11, 31).

Histological, immunohistochemical, and in situ hybridization techniques revealed that three modes of ossification occur during distraction osteogenesis (10, 18, 32-36). Typical endochondral bone formation can occur in the early stage of distraction, as is the case in fracture healing, but intramembraneous

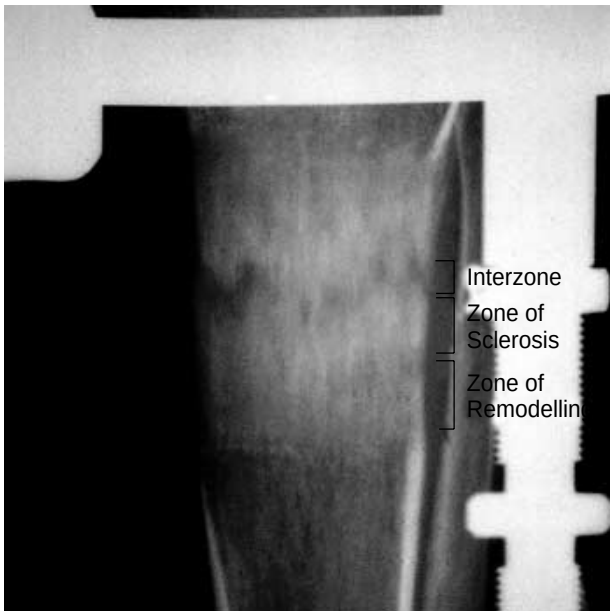

Fig. 3. Three distinct zones are seen in a human tibial lengthening: a central radiolucent zone (interzone), a zone of increased bone density (zone of sclerosis), and a zone with low density (zone of remodeling).

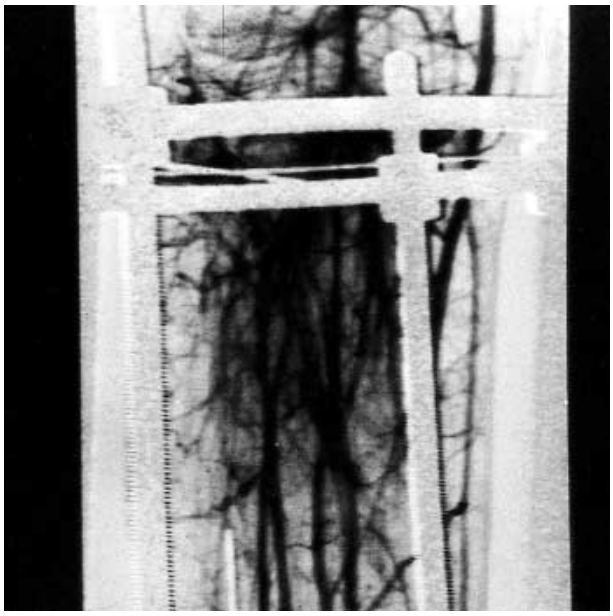

Fig. 4. An angiogram during the distraction phase in a human tibial lengthening showing a significant increase of vascularity, suggestive of active angiogenesis and vasculogenesis.

bone formation is the predominant mechanism of ossification, particularly in the later stages. Yasui et al. (36) proposed a third mechanism of ossification, so called 'transchondroid bone formation'. They observed that the chondroid bone, a tissue intermediate between bone and cartilage, was formed directly by chondrocyte-like cells, with transition from fibrous tissue to bone occurring gradually and consecutively without capillary invasion. In situ hybridization using digoxigenin-11-UTP-labeled complementary RNAs showed that the chondroid bone cells temporarily expressed type-II collagen mRNA. They did not show the classical morphological characteristics of chondrocytes, but were assumed to be young chondrocytes undergoing further differentiation into bone-forming cells. Li et al. also (37) observed, in a rabbit model of distraction osteogenesis, that acid phosphatases were found within the cartilage matrix in some of the cartilage/bone transitional regions and that collagen type 1 mRNA and collagen type 2 protein were found together in some of the marginal hypertrophic chondrocytes.

COUPLING BETWEEN ANGIOGENESIS AND MINERALIZATION

Several reports document a significant increase of blood supply during distraction osteogenesis based upon microangiographic, vascular corrosion casting (18), and quantitative scintigraphic studies (12, 20) (Fig. 4). Regional perfusion studies have demonstrated increased blood flow, up to 10 times greater than in controls, during the distraction period at the site of bone formation. Although these increased perfusion levels do not seem to be prolonged by an increase in the period of distraction, blood flow in the range of 3 times that of control levels persists for at least 17 weeks after corticotomy (5-7). The increased level of blood flow is also observed in the distant site of bone formation in the same segment (7). Hematopoietic function increases in accordance with the increase of blood flow (3).

It has been known that bone development and remodeling depend on complex interactions between bone-forming osteoblasts and other cells present within the bone microenvironment, particularly vascular endothelial cells (ECs) that may be pivotal members of a complex interactive communication network in bone. This may be the true in distraction osteogenesis. Villars et al. (38) investigated the interaction between human umbilical vein ECs and human bone marrow stromal cells. They reported that cell differentiation analysis performed with different cell culture models revealed that alkaline phosphatase activity and type I collagen synthesis were increased only by the direct contact of the ECs with stromal cells. A dye coupling assay demonstrated a functional coupling between the ECs and stromal cells. Some authors (39, 40) have suggested that either vascular ECs or pericytes differentiate into osteoblasts or precursor cells, which means that vessels could directly participate in bone formation. Wound trauma causes mobilization of hematopoietic cells, including pluripotent stem or progenitor cells in spleen, bone marrow, and peripheral blood. Circulating and/or bone marrow-derived endothelial progenitor cells (EPCs) may home to sites of active "angiogenesis (neovascularization from preexisting vessels)" and there differentiate into ECs, in a so called "vasculogenesis" process (41-43) during

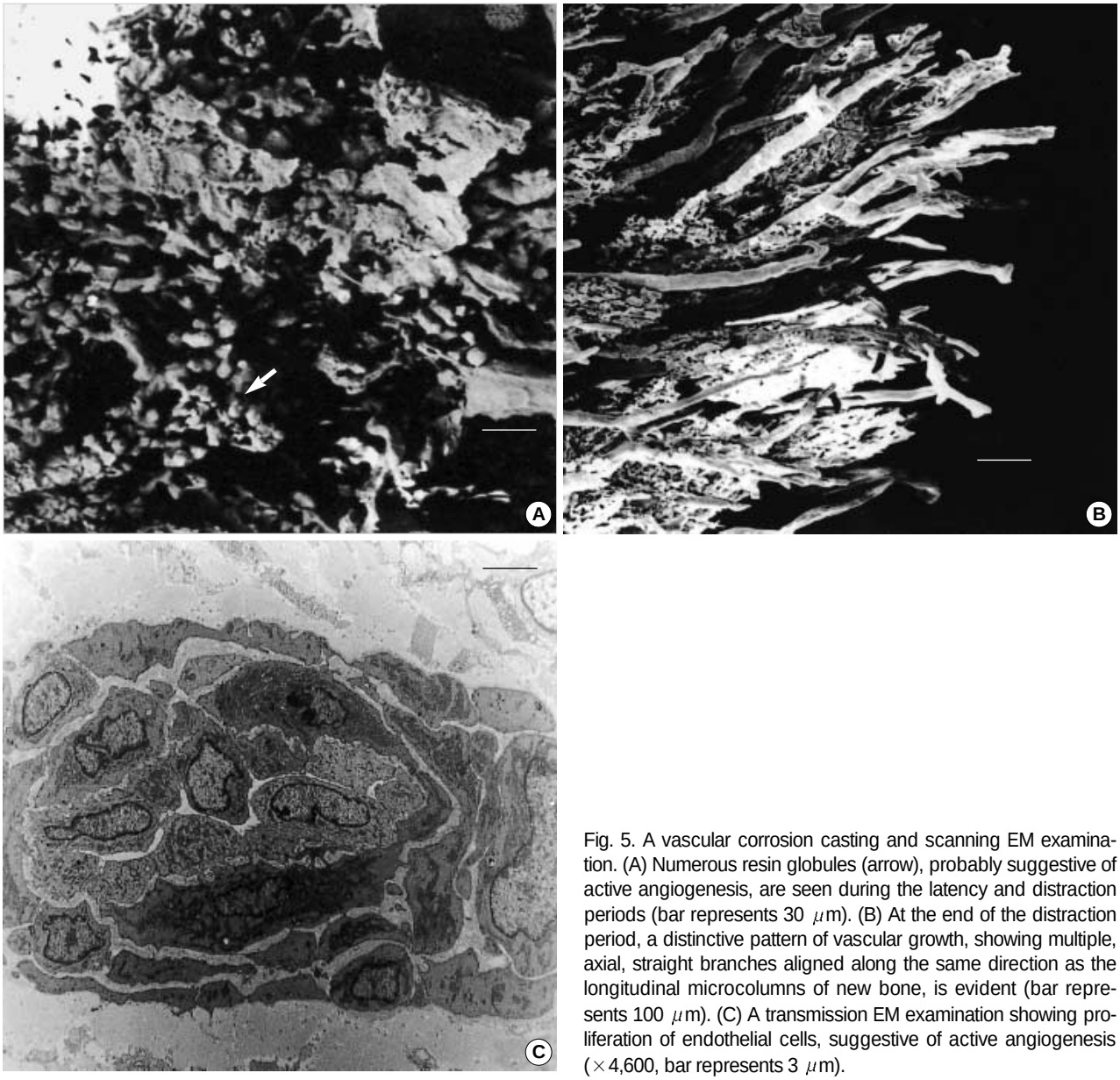

Fig. 5. A vascular corrosion casting and scanning EM examination. (A) Numerous resin globules (arrow), probably suggestive of active angiogenesis, are seen during the latency and distraction periods (bar represents 30 μm). (B) At the end of the distraction period, a distinctive pattern of vascular growth, showing multiple, axial, straight branches aligned along the same direction as the longitudinal microcolumns of new bone, is evident (bar represents 100 μm). (C) A transmission EM examination showing proliferation of endothelial cells, suggestive of active angiogenesis ($\times$4,600, bar represents 3 μm).

distraction osteogenesis.

Histological and ultrastructural studies have demonstrated that vessels of uniform diameter that extend from each surface (periosteal and endosteal) of the host bone toward but not across the FIZ, are oriented in the same direction as the microcolumns of new bone (5, 6, 10, 11, 18, 22). Immunohistochemical analysis also has provided evidence of active angiogenesis with the identification of two constituents of vascular basement membrane laminin and type-IV collagen (23). Capillary precursors are found in the FIZ ahead of the PMF (44). Ilizarov (3) observed two different types of capillary formation, i.e., sinusoidal capillary and transport capillary, at the site of angiogenesis on transmission EM. We examined the spatial structure of vascular development in a rat model of distraction osteogenesis using a vascular corrosion cast and scanning EM (18). Our study clearly implicated a close temporal and spatial relationship between periosteal and medullary vascular proliferation and mineralization in the distraction gap. Active angiogenesis occurred during the latency and distraction period (Fig. 5A). During the early distraction period, periosteal vessel proliferation was more conspicuous than that of endosteal vessels. Early formation of the vascular network, apparently derived from the medullary sinusoids and the periosteal vessels, was noticed in the corresponding area of subperiosteal new bone formation. This observation may support the suggestion that the periosteum not only fulfills many important functions for vascular supply but also provides osteoblast lineage including osteoprogenitor cells (16,

18, 40). At the end of the distraction period, the transitional zone between the FIZ and the host bone was richly vascularized from its periosteal and endosteal surfaces towards the FIZ, which itself lacked vascularization. A distinctive pattern of vascular growth was observed, showing multiple, axial, straight branches aligned along the same direction as the longitudinal microcolumns of new bone (Fig. 5B). During the consolidation period, the periosteal and medullary vascular networks were completely connected to each other at the distraction site including the FIZ, and the distraction gap was eventually filled with regenerating osteogenic tissue.

SKELETAL, CELLULAR, AND MOLECULAR BASES OF DISTRACTION OSTEOGENESIS

Rapid progress in skeletal cellular and molecular biology has led to the identification of many signaling molecules associated with the formation of skeletal tissues. Recent work has focused on the mechanisms by which growth and differentiation factors regulate the process of regenerate bone formation and maturation.

Collagen and Osteogenic Markers

Mechanical tension-stress modulates cell shape and phenotype, and stimulates the expression of the mRNA for bone matrix proteins, as well as the assembly of collagen and mineralization during distraction osteogenesis (45-47). Many experimental data indicate that during active distraction, collagen type-I is expressed in the periosteum and the PMF, whereas collagen type-II transcripts are localized to discrete regions on the periosteal surfaces, immediately adjacent to the osteotomy ends. Collagen type-II transcripts are usually not detected in the FIZ. During the maturation phase, cells within the FIZ express collagen type-I and exhibit abundant alkaline phosphatase activity, suggestive of terminal differentiation. Alkaline phosphatase activity is detected in the endosteal and periosteal surfaces of the bone ends, and tartrate-resistant acid phosphatase staining reveals that osteoclasts remodel the bone regenerate as it forms (48). The continuous evolution of the tensile behavior of the newly formed osseous tissue correlates with the plasma bone-specific alkaline phosphatase activities (3).

Meyer et al. (32-34, 49, 50) reported that in contrast to bone-like apatitic formation of crystals at a physiological magnitude, hyperphysiological magnitudes of strain (fast distraction rate) resulted in a reduced expression rate of osteocalcin (OC) and osteonectin (ON) that was paralleled by a significant loss of crystal (fewer but larger crystals) formation. The variety of cell types expressing mRNA encoding bone matrix proteins in distraction osteogenesis is much greater than that detected in the embryonic bone formation and fracture healing process (35). Perrien et al. (46) observed the expression of osteopontin (OPN), a multifunctional matricellular protein believed to play a key role in wound healing and cellular response to mechanical stress. They found that fibroblast-like cells within the FIZ exhibited intermittent low levels of OPN, although no relationship was observed between OPN and proliferation. In areas of transchondral ossification, OPN expression was very high in the morphologically intermediate oval cells. During intramembranous ossification, osteoblasts appeared to exhibit a bimodal expression of OPN. Specifically, proliferating pre-osteoblasts expressed OPN, but it was not detected in the post-proliferative pre-osteoblasts and osteoblasts that border the new bone columns. Finally, intracellular OPN was detected in virtually all of the mature osteoblasts/osteocytes within the new bone columns. They concluded that early expression of OPN may facilitate pre-osteoblastic proliferation and migration, while later downregulation may be necessary for hydroxyapatite crystal formation. Sato et al. (35) observed in a rat model that during active distraction, the chondrocytes were stretched along the tension vector, became fibroblast-like in shape, and expressed OPN, OC, and alkaline phosphatase mRNAs. As distraction advanced, the cartilaginous callus was progressively replaced by bony callus through endochondral ossification, and thereafter new bone was formed directly by intramembranous ossification. OPN mRNA was detected in preosteoblasts and osteoblasts at PMF. ON, matrix gla protein (MGP), and OC mRNAs appeared early in the differentiation stage. Moreover, the levels of OPN, ON, MGP, and OC mRNA expression markedly increased during the distraction phase.

Growth Factor and Cytokine

Increasing evidence indicates that there are critical regulators of cellular proliferation, differentiation, extracellular matrix biosynthesis and mineralization. Several authors (16, 17, 19, 51, 52) investigated the effect of mechanical tension-stress on gene expression of bone morphogenetic proteins (BMPs) and other growth factors including insulin-like growth factor (IGF), basic fibroblast growth factor (bFGF), transforming growth factor beta (TGF-beta), growth/differentiation factor 5 (GDF-5), and vascular endothelial growth factor (VEGF) using immunohistochemical staining, northern blot analysis, and in situ hybridization.

Several authors (19, 51, 52) hypothesized that BMPs play an important role in the signaling pathways that link the mechanical forces created by distraction to biological responses. The BMP genes appear to participate in regulating bone and cartilage formation in distraction osteogenesis. Li et al. (51) studied the presence and localization of BMP-4 mRNA in the regenerating tissues produced in a rabbit model of tibial lengthening by distraction osteogenesis. They found that, as in fracture repair, the BMP-4 gene was expressed by less differentiated osteoprogenitor cells (fibroblastic mesenchymal cells and preosteoblasts), and not by fully differentiated osteoblasts. BMP-4 gene expression was localized in callus-forming tissue (muscle, periosteum) during callus formation. Sato et al.

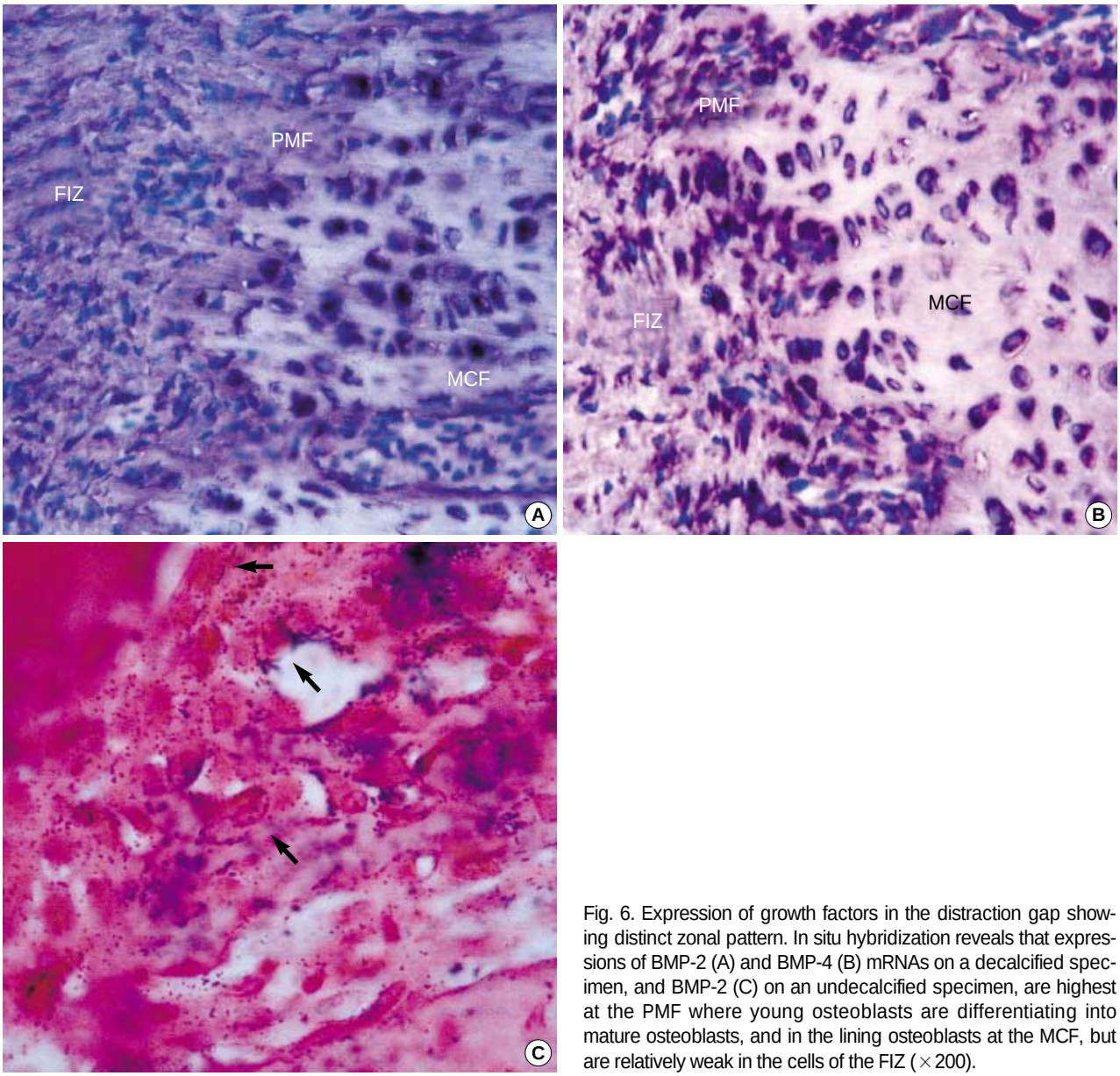

Fig. 6. Expression of growth factors in the distraction gap showing distinct zonal pattern. In situ hybridization reveals that expressions of BMP-2 (A) and BMP-4 (B) mRNAs on a decalcified specimen, and BMP-2 (C) on an undecalcified specimen, are highest at the PMF where young osteoblasts are differentiating into mature osteoblasts, and in the lining osteoblasts at the MCF, but are relatively weak in the cells of the FIZ (×200).

(19) used in situ hybridization and Northern blot analysis in a rat model of femoral lengthening (latency period of 7 days, followed by distraction for 21 days at a rate of 0. 25 mm/12 hr) to examine the expression of BMP-2, BMP-4, BMP-6, BMP-7, and GDF-5. As distraction commenced, the callus elongated and expression of BMP-2 and BMP-4 mRNAs was markedly induced at this stage. Their signals were detected widely among chondrogenic and osteogenic cells and their precursor cells sustained mechanical tension-stress at the FIZ. BMP-6 and GDF-5 mRNAs were detected exclusively in chondrogenic cells at both ends of the FIZ, where endochondral ossification occurred. As distraction advanced, the cartilage was progressively resorbed from both ends and new bone was formed directly by intramembranous ossification. The signals of BMP-6 and GDF-5 mRNA declined by this stage, while those of BMP-2 and BMP-4 were maintained at a high level for the duration of distraction. Neither BMP-2, BMP-4, BMP-6, nor GDF-5 was expressed at the consolidation stage. The signals of BMP-7 were not detected throughout the experiment. They concluded that excellent and uninterrupted bone formation during distraction osteogenesis is due to enhanced expression of BMP-2 and BMP-4 genes that can induce in situ bone formation by paracrine and autocrine mechanisms. In contrast, Rauch et al. (52) investigated, in a rabbit model, the temporal and spatial expression of BMP-2, -4, and -7 proteins during distraction osteogenesis using immunohistochemistry. Staining for BMP-2, -4, and -7 was evident before distraction was applied and was mainly localized to mesenchymal cells and

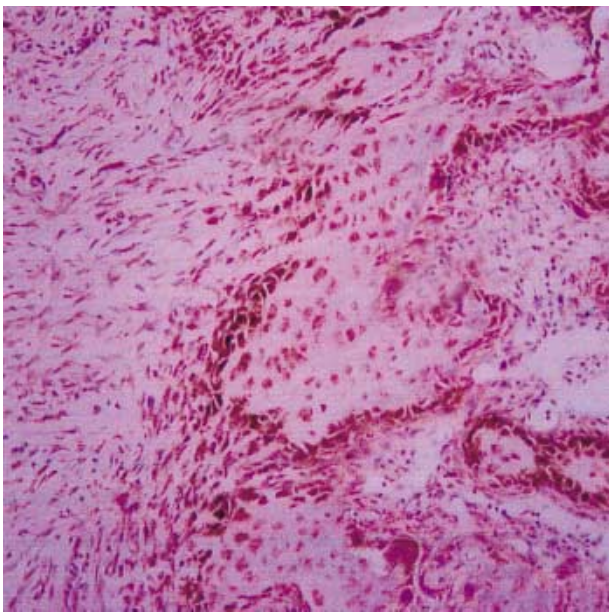

Fig. 7. Immunohistochemical staining reveals that the expression of VEGF is highest at the PMF where young osteoblasts are differentiating into mature osteoblasts. Positive VEGF expression is also observed in the lining osteoblasts at the MCF, but is relatively weak in the cells of the FIZ. VEGF positivty is also observed in the osteoblasts in the endosteum and periosteum, but not in the osteocytes, of the host bone. The localization and pattern of VEGF expression correspond to those of BMP-2 and BMP-4 expression (×100).

osteoblastic cells in the periosteal region. After distraction commenced, the cells in the typical FIZ, resembling fibroblasts and chondrocytes, showed intense staining for all three BMPs. This high level of expression was maintained during the entire distraction phase and then gradually disappeared during the consolidation phase. Our observations on the expression of BMP-2 and BMP-4 using the in situ hybridization technique (16) were similar to those reported by previous authors (19, 51, 52). Expression of BMP-2 and BMP-4 mRNA was highest at the PMF where young osteoblasts were differentiating into mature osteoblasts, and in the lining osteoblasts at the MCF, but was relatively weak in the cells of the FIZ (16) (Fig. 6).

The role of TGF-beta, IGF-1 and bFGF, the expressions of which are increased by mechanical strain, was also investigated (53-57). These reports indicated that during the distraction period, the cells of osteoblastic lineage and the mesenchymal cells on the newly formed trabecular bone and PMF express bFGF. Farhadieh et al. (53) speculated that concentrated presence of IGF-1 and bFGF in the distracted region may account for osteoblast proliferation and formation from precursor mesenchymal cells. The sections obtained from groups distracted at faster rates showed a stronger presence of the growth factors examined by more intense staining (53). The expression of bFGF during the distraction period was stronger than that during the latency and consolidation periods. However, some osteoblasts continued to express bFGF in the consolidation period (58). There was diffuse presence of TGF-beta throughout the lengthened region corresponded with the process of intramembranous ossification. Liu et al. (55) observed that TGF-beta1 staining was predominantly localized to the osteotomized bone edges, periosteum, surrounding soft tissues, and residual inflammatory cells. Osteoblasts and fibroblast-like cells in the FIZ, along with osteoblasts in all zones including the osteoid seam, were stained for TGF-beta and its receptor. IGF-I could be detected everywhere. The increased level of TGF-beta1, 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 OC production. Tavakoli et al. (57) suggested that bFGF, IGF-1, and TGF-beta may play different roles in the remodeling phase of distraction osteogenesis. Cillo et al. (59) determined the effect of continuous cyclic mechanical stretch as a fundamental event in distraction osteogenesis, on the expression of three bone growth factors, TGF-beta1, IGF-1, and bFGF, and two cytokines, interleukin-1 (IL-1) and interleukin-6 (IL-6), in human osteoblast-like cells, SaOS-2, which are capable of forming a ground substance and mineralizing it. They concluded that tensile stretch on osteoblast-like cells alters the local regulation of bone formation and thereby increases the expression of bone growth factors, whereas catabolic cytokines are unaffected.

We examined the temporal and spatial expression of VEGF mRNA by in situ hybridization and immunohistochemistry in a rat model of tibial lengthening by distraction osteogenesis (7 days latency period, followed by 14 days of distraction period at a distraction rate of 0.5 mm/day in two increments) (17). The animals were killed at postoperative 1st, 2nd, 3rd, and 4th weeks. VEGF mRNA was expressed throughout the whole procedure, and the relative dominance of splicing variants ($VEGF_{164}$,$VEGF_{188}$,$VEGF_{205}$) varied during distraction osteogenesis. VEGF expression of both mRNA and protein was highest at the PMF where young osteoblasts were differentiating into mature osteoblasts. Positive VEGF expression was also observed in the lining osteoblasts at the MCF, but was relatively weak in the cells of the FIZ. VEGF positivity was also observ- ed in the osteoblasts in the endosteum and periosteum, but not in the osteocytes, of the host bone. The localization and pattern of VEGF expression corresponded to those of BMP-2 and BMP-4 expression (6) (Fig. 7).

FACTORS AFFECTING ANGIOGENESIS AND MINERALIZATION

Many mechanical and biological variables appear to affect not only the differentiation of osteoblasts and chondrocytes within the regenerate originating from the same pool of progenitor cells, but also vascular proliferation and blood supply

for angiogenesis and mineralization during distraction osteogenesis. First of all, it has been shown that all these processes are influenced strongly by mechanical loading on the local tissue. Carter et al. (60) proposed some of the mechanobiological principles that are thought to guide the differentiation of mesenchymal tissue into bone, cartilage, or fibrous tissue during the initial phase of regeneration. They concluded that for intermittently imposed loading in the regenerating tissue: (a) direct intramembranous bone formation is permitted in areas of low stress and strain; (b) low to moderate magnitudes of tensile strain and hydrostatic tensile stress may stimulate intramembranous ossification; (c) poor vascularity can promote chondrogenesis in an otherwise osteogenic environment; (d) hydrostatic compressive stress is a stimulus for chondrogenesis; (e) high tensile strain is a stimulus for the net production of fibrous tissue; and (f) tensile strain with a superimposed hydrostatic compressive stress will stimulate the development of fibrocartilage. From the mechanobiological point of view, poor osteotomy, frame instability, and a high distraction rate (10) may disturb vascularization and local blood supply to regenerating tissues, thereby causing delayed bone healing. Instability due to fixator constructs that allow excessive motion between the distracted bone segments (9, 10) may lead to local hemorrhage and the formation of islands of cartilage. Local dysvascularity of one or both distracted surfaces can occur secondary to thermal necrosis; for example, from uncooled power tools (61) or from a high-energy injury such as that resulting from a widely displaced or comminuted osteotomy (1). The resultant ischemic tissue may fail to form bone and could result in a fibrous or cartilaginous non-union (5, 6, 8). Frierson et al. (61) observed histologically that when osteotomy was carried out with oscillating saw, bone consolidation was delayed as compared to the osteotomy performed by corticotomy or drill-osteoclasis. Weight-bearing appears to affect the speed of regenerate bone formation and maturation. Radomisli et al. (46) observed that there was more new bone in the distraction gap of the weight-bearing animals than in the non-weight-bearing animals. BMP-2 and BMP-4 expression, as well as the messages for collagen type 1 and OC, were more abundant in tissue from the weight-bearing animals; whereas collagen type 2 was higher in the non-weight-bearing animals.

Many reports (33, 34, 54, 62) indicate that the magnitude (strain) rather than the frequency of mechanical loading controls the differentiation of bone cells and the subsequent formation of bone tissue. Neither the rhythm of distraction nor the relative lengthening appears to significantly influence any morphometric parameters evaluated. A faster distraction rate may result in formation of chondroid or fibrous tissue instead of osseous tissue in the distraction gap (32-34, 37, 44, 49, 50, 63).

Of the biologic factors, age is one of the most important determinants for bone formation. Clinical experiences indicate that radiographic findings in older patients demonstrate significant delays in mineralization during distraction osteogenesis. This may be related to the fact that retardation of neovascularization in older age groups appears in part to result from reduced expression of VEGF and inherent limitations imposed by a less-responsive EC substrate (41). Aronson (8) reported, on the basis of over 100 clinical cases of patients ranging in age from 18 months to 49 yr, that regenerated bone formed at an average rate of 213 μm/day in adults and 385 μm/day in children. He and his associates (9) also investigated the effect of aging on distraction osteogenesis in rats with mid-diaphyseal osteotomy. They observed that in 4-month-old rats, proliferating cell nuclear antigen (PCNA)-immunostained cells were organized along the PMF extending across both periosteal and endosteal surfaces. In 24-month-old rats, PCNA-positive cells were organized in zones along the periosteal new bone fronts only, and were irregularly scattered throughout the endosteal gap within a fibrovascular non-ossifying matrix, indicative of a relative deficit in endosteal bone formation. Lumpkin et al. (64) studied the impact of total enteral nutrition on distraction osteogenesis in a rat model. They observed that this form of nutritional support dramatically increased the mineralized bone formed over the 20-day distraction period, and accelerated entry into the remodeling phase of consolidation. The effects of other factors such as administration of pharmaceuticals, e.g. methotrexate (43), steroids, and smoking, on regenerate bone formation need to be fully investigated in the future.

EXPERIMENTAL INVESTIGATIONS TO PROMOTE REGENERATE FORMATION AND MATURATION

In order to enhance regenerate bone formation and maturation, and thereby to shorten treatment time, the use of adjunctive modalities, including the transplantation of progenitor cells, administration of growth factors, hormones, and bisphosphonate, and the application of demineralized bone matrix, calcium sulfate, and electrophysiological tools has been extensively investigated (Table 1). Recent experimental works implicate that bone marrow-derived mesenchymal stem cells including progenitor cells of osteoblast and ECs, can be used for transplantation to enhance angiogenesis and mineralization. Tsubota et al. (65) reported that transplantation of osteoblast-like cells derived from the rabbit's tibial periosteum to the centers of distracted callus, immediately after distraction had been terminated, promoted maturity of the distracted callus. They observed that 2 weeks after transplantation, the transaxial area ratio at the center of the distracted callus, and the bone mineral density (BMD) were significantly higher in the transplanted group, by 21% and 42%, respectively, than in the control groups. Mechanically, the callus in the transplanted group tended to be stronger. Hagino et al. (66) reported that grafting with demineralized bone matrix allowed for satisfactory bone healing at a faster rate than normal, even at a distraction rate of 2-3 mm/day. Likewise, the application

Table 1. Experimental investigations to promote regenerate bone healing during distraction osteogenesis

Cell therapy: Transplantation of osteoblast-like cells to the distracted callus (65)
Demineralized bone matrix (66)
Calcium sulfate (67)
Bisphosphonate (68, 69)
Hormones: Recombinant growth hormone (47, 70, 71); 2-beta-(3-hydroxypropoxy)-1alpha, 25 dihydroxyvitamin D3 (ED-71) (72)
Growth factors: bFGF (45, 50); IGF (73); VEGF; TGF-beta 1* (74, 75)
Low-intensity pulsed ultrasound (77, 79)
Electrical stimulation: Direct current (80); Capacitively coupled electromagnetic field* (81)

* indicates negative outcomes.

of resorbable calcium sulfate material (67) to newly distracted bone increased the rate of osteogenesis and consolidation. The administration of bisphosphonates, pamidronate (68), and zoledronic acid (69) can improve the BMD, BMC, and mechanical properties of a bone undergoing distraction osteogenesis. Little et al. (68) reported that pamidronate had a markedly positive effect in increasing the osteoblastic rimming and mineralization of regenerate bone in rabbits, showing increased formation of bone around the pin sites and an increase in the cortical width of the bone adjacent to the regenerate. It reduced the disuse osteoporosis normally associated with lengthening when an external fixator is used, and increased the amount and density of the regenerate bone.

The application of recombinant homologous (47) and species-specific (70, 71) growth hormone (GH) has also been proved to show stimulating effect on regenerate bone healing without changing the callus microstructure. Raschke et al. (47) administered 100 μg r-pGH per kg bodyweight per day in the micropigs for tibial lengthening (2 mm daily over a period of 10 days followed by 10 days of consolidation before sacrifice). Final regenerate torsional failure load was 131% higher and ultimate torsional stiffness was 231% higher in the treatment group than in the control group. On the other hand, Yamane et al. (72) investigated the effect of 2-beta-(3-hydroxypropoxy)-1 alpha, 25-dihydroxyvitamin D3 (ED-71) on the modeling of bone in the tibial lengthening of rabbits. Following osteotomy, ED-71 (0.05 μg/kg) was administered subcutaneously twice a week. They concluded that ED-71 increases callus volume during the early period after the completion of lengthening, resulting in thick cortical bone formation.

Although the use of growth factors is rapidly expanding, the application to the human subjects is still under development. Several authors (45, 50) investigated the stimulation of bone formation by recombinant bFGF during distraction osteogenesis. Okazaki et al. (50) investigated the effects of a single local injection of recombinant human bFGF (200 μg of bFGF in 150 μLof saline solution) in rabbits. Injection of bFGF into the center of the distracted callus on the final day of distraction increased bone formation at the distracted site. A significant effect on BMC at the callus was observed as early as 2 weeks after injection, which increased about twofold at 5 weeks after a normal remodeling process. The application of bFGF was proved to be effective in enhancing regenerate bone formation in the distraction osteogenesis of irradiated bone (45). Similarly, exogenous IGF-1 has a positive influence on osteoblastic activity during distraction. Stewart et al. (73) reported that recombinant IGF-1 infusion significantly enhanced osteoblastic activity at distraction rates of both 1mm/day and 3 mm/day in rabbit's mandible, and resulted in bony union at the latter rate. In contrast to the positive effect of IGF and bFGF, locally applied TGF-beta1 did not have a beneficial effect. Rauch et al. (74) reported their results in a rabbit model (7 days of latency, followed by 3 weeks of distraction at a rate of 0.25 mm/12 hr for 3 weeks) with TGF-beta1 (0, 10, 20, and 40 ng/day) administered, from the commencement of distraction, to the site of osteotomy via a subcutaneously implanted miniosmotic pump. They observed that while TGF-beta1 treatment had no detectable effect on BMD or histologically determined bone volume in the distraction gap, it increased the amount of fibrous tissue in the callus region. Load to failure in uniaxial tension tended to be lower in TGF-beta1-treated animals. Sciadini et al. (75) also observed that the one-time administration of TGF-beta retarded the formation of a stable, united regenerate. We believe that VEGF is another candidate growth factor that can enhance regenerate bone healing, by means of promoting the proliferation and differentiation of osteoblast lineage cells and EPCs. In the future, gene therapy may offer ways of enhancing bone formation, as in fracture healing, by altering the expression of desired growth factors and extracellular matrix molecules. Spector et al. (76) proposed a method utilizing adenovirus to deliver gene products in healing osseous tissues. However, the elucidation of suitable candidate genes for therapeutic intervention necessitates thorough investigation of the endogenously expressed patterns of growth factors during normal fracture repair and distraction osteogenesis.

Several authors have investigated the effects of the application of ultrasound (77) and electrical stimulation (21, 78) on regenerate bone formation in distraction osteogenesis. Clinically, prospective, randomized, and double-blind trials showed the efficacy of low-intensity, ultrasound beam stimulation in the acceleration of fracture healing, with a significant decrease in the time to healing. As previously observed in a model of fracture repair, the positive effects of the low-intensity ultrasound beam during distraction osteogenesis were reported (77, 79). Shimazaki et al. (79) claimed that ultrasound can accelerate bone maturation in distraction osteogenesis in rabbits, even in those in states of poor callotasis. On the other hand, Hagiwara et al. (80) investigated the effect of electrical stimulation on distraction osteogenesis of rabbit's mandible. They applied direct current electrical stimulation (10 μA) to two of the screws used as electrodes during the distraction phase, and observed that the new bone formation 10 and 20 days

after distraction was greater in the electrical stimulation group than in the control group. Ten and 20 days after distraction, image analysis and analysis of BMD in areas of newly formed bone indicated that there was a greater amount of new bone formation in the stimulation group than in the control group. They concluded that electrical stimulation during gradual distraction promotes new bone formation in the early retention period in a rabbit model. Contrary to the positive effect of direct current on regenerate bone formation, the capacitively coupled electrical stimulation demonstrated negative effects on regenerate bone healing (81).

FUTURE DIRECTIONS

Rapid progress in skeletal cellular and molecular biology has led greater understanding of the biology of distraction osteogenesis. Recent advances in the identification of many signaling molecules associated with the formation of skeletal tissues are promising. However, further in-depth basic research should be conducted to: (a) elucidate the exact molecular mechanisms by which growth and differentiation factors regulate the process of regenerate bone formation and maturation including the mechanism of proliferation and differentiation of mesenchymal stem cells to osteoblast lineage cells and endothelial cells; (b) determine the origin of the PCNA-positive cells in the distraction gap; (c) determine the mechanism of biological and biomechanical variables affecting angiogenesis and mineralization; and (d) develop the most effective and efficient modality, including the use of bioactuators and/or biomodulators, to accelerate regenerate bone healing and remodeling, while taking into account the close relationship between angiogenesis and mineralization. It is our belief that with the advent of effective and efficient bioregulators and modulators, the development of distraction osteogenesis will proceed to a level enabling the treatment of severe musculoskeletal conditions.

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