Increased Lengthening Rate Decreases Expression of Fibroblast Growth Factor 2, Platelet-Derived Growth Factor, Vascular Endothelial Growth Factor, and CD31 in a Rat Model of Distraction Osteogenesis

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Background: The rate of lengthening has a profound impact on bone regeneration during distraction osteogenesis. Rapid distraction can delay or completely inhibit union, whereas distracting too slowly may lead to premature consolidation. However, the mechanisms responsible for retardation of healing due to rapid distraction have not been elucidated. This study explored whether rapid distraction alters the expression of certain angiogenic growth factors, in particular, fibroblast growth factor 2 (FGF-2), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF-AA), and subsequent new vessel formation as evidenced by platelet endothelial cellular adhesion marker expression (CD31), an indicator of vascular budding.

Methods: Unilateral femoral lengthenings were performed in 60 male Sprague-Dawley rats using a protocol that involved a 7-day latency period and distraction rates of either 0.5 (slow distraction) or 1.5 mm/d (fast distraction) for a total of 7.0 mm of lengthening. Animals were euthanized on postoperative days 8, 10, 12, 14, and 21 (n = 6 per time point and distraction rate). Expression of FGF-2, VEGF, PDGF-AA, and CD31 was characterized immunohistochemically.

Results: Cellular staining of FGF-2, PDGF-AA, VEGF, and CD31 was reduced on days 8 to 12 in the regenerate of the fast-distraction animals compared with the slow-distraction animals. Staining of all growth factors was weak on days 14 and 21 at the slow rate and absent at the fast rate. Regardless of time point, a similar spatial localization of growth factor expression was observed at the 2 rates of distraction.

Conclusions: The reduced expression of angiogenic growth factors and CD31, a marker of new vessel formation, indicates that the angiogenic cascade and new vessel formation required for effective bone healing is disrupted at a distraction rate of 1.5 mm/d in a rat model of limb lengthening.

Clinical Relevance: Delayed bone healing with rapid distraction may be due in part to decreased cellular signaling required for angiogenesis. It may be possible to improve bone healing at increased distraction rates with the appropriately timed administration of growth factors.

Key Words: distraction osteogenesis, angiogenesis, limb lengthening, growth factor, delayed union, nonunion

The rate of lengthening has been found to negatively impact bone regeneration in several mandibular and long-bone animal models.1,5 For example, rapid distraction has been shown to inhibit angiogenesis and disrupt bone regeneration in a rabbit midtibial distraction model.3 Similarly, Paccione et al3 found distraction rates of 1.0 mm/d in a rat mandible disrupted angiogenesis and led to poor regenerate bone, whereas, 0.5 mm/d fostered excellent regenerate bone. Clinically and experimentally, distraction rates greater than 1.0 mm/d has been found to injure the periosteum and developing blood vessels, delaying union or leading to complete nonunion.2,6

At the tissue level, rapid distraction is typified by the early deposition of loosely organized granulation tissue containing few developing blood vessels.7 As distraction progresses, granulation tissue, fibrocartilage, and cysts fill the distraction gap, with only sparse development of regenerate bone. Reduced osteoblast activity and the presence of cartilage and cysts delay or inhibit bone formation across the distraction gap, leading to nonunion.1,8,9

Several studies have identified growth factors in the distraction zone that are critical for angiogenesis and subsequent bone regeneration during distraction osteogenesis.3,10-13 Platelet-derived growth factor (PDGF-AA), fibroblast growth factor 2 (FGF-2), vascular endothelial growth factor (VEGF), and vascular indicator platelet endothelial cellular adhesion marker (CD31) have been identified during distraction osteogenesis.11,14-21 The chemotactic and mitogenic effects of growth factors on fibroblasts, osteoblasts, and endothelial cells stimulate and modulate angiogenesis, cell migration and proliferation, osteogenesis, and bone remodeling.11,20,22,23 Decreased expression of necessary growth factors can retard or completely inhibit bone regeneration and subsequent healing.7,24

In this study, we hypothesized that rapid distraction would lead to decreased expression of cellularly derived angiogenesis-related growth factors FGF-2, VEGF, and PDGF-AA, and the vascular marker platelet endothelial cellular adhesion marker 1/CD31, compared with the levels
expressed during slower distraction. Specifically, we examined the temporal and spatial expression of these angiogenic and osteogenic cytokines in a rat limb-lengthening model.

METHODS

Animal Model

After approval by the Rhode Island Hospital animal welfare committee, unilateral femoral lengthenings were performed in 60 mature, male Sprague-Dawley rats (420–560 g, age, <6 months). The rats were anesthetized with an intraperitoneal cocktail of ketamine (50 mg/kg; Abbott Laboratories, Chicago, IL) and Domitor (0.5 mg/kg; Orion Pharma, Orion Corp, Finland), and a subsequent preoperative dose of ceftazolin (20 mg/kg; SmithKline Beecham, Pittsburgh, PA) was administered for infection prophylaxis. The lateral aspect of the right hindlimb was shaved, scrubbed with Betadine, and sterily draped. Through an anterolateral longitudinal incision, 2 pairs of 1.0-mm-diameter holes, positioned with the use of a specially designed guide, were drilled through the proximal and distal femoral cortices. The holes were tapped, and 2 partially threaded 1.25-mm Kirschner wires were inserted. The lateral ends of the K-wires were affixed to a specially designed adjustable monolateral external fixator.25

After application of the external fixator, a periosteal-sparing osteotomy was made at the middiaphysis by partially cutting the bone with an oscillating saw and then completing the osteotomy with an osteotome. The surgical incision was closed with interrupted sutures, and the animals were returned to the cages and allowed unrestricted activity. Buprenex (0.3 mg/kg; Reckitt and Colman, Richmond, VA) was given the first 2 days postoperatively for analgesia. After a 7-day latency period, the femurs were distracted with twice-daily lengthenings of either 0.25 (healing/slow rate; n = 30) or 0.75 mm (nonunion/fast rate; n = 30) for a total of 7.0 mm of distraction in each group. Animals were euthanized on postoperative days 8, 10, 12, 14, and 21 (n = 6 per time point).23,21

Tissue Processing and Immunohistochemistry

The distracted femurs were harvested en bloc, with a small margin of muscle, and fixed in formalin. The specimens were decalcified with EDTA and embedded in paraffin. Serial 5.0-μm sagittal sections were obtained for hematoxylin and eosin staining and subsequent immunohistochemical analysis for FGF-2, VEGF (recognized polymorphs B, C, and D), PDGF-AA, and CD31.

For immunohistochemistry, the slides were deparaffinized with xylene and rehydrated with a graded series of ethanol solutions, followed by washing in distilled water for 5 minutes. Antigen unmasking was accomplished by incubating at 95 °C in 10 mM sodium citrate buffer (pH 6). To quench endogenous peroxidase activity, the slides were treated with a 3% hydrogen peroxide solution in methanol for 30 minutes. Nonspecific binding was blocked with 1.5% goat serum (PDGF-AA, FGF-2, and VEGF) or horse serum (CD31).

Three separate slides were incubated at 4 °C overnight with rabbit polyclonal PDGF-AA, FGF-2, VEGF, or goat polyclonal CD31 primary antibody (1:150; Santa Cruz Biotechnology, Santa Cruz, CA) diluted in 1.5% blocking serum in a high-humidity chamber. The antibodies were diluted per the manufacturers’ recommendations. After incubation with primary antibody, the specimens were washed in phosphate-buffered saline (PBS), followed by incubation for 30 minutes at room temperature with biotinylated secondary antibody; goat antirabbit immunoglobulin G for PDGF-AA, FGF-2, VEGF, and horse antigoat immunoglobulin G for CD31 (ABC Immunoperoxidase Staining System, Santa Cruz Biotechnology). The slides were then washed in PBS, incubated with avidin-biotinylated horseradish peroxidase for 30 minutes, washed in PBS again, and then incubated with 2 drops of peroxidase substrate. After rinsing with distilled water, the slides were counterstained with Gill hematoxylin, rinsed in distilled water, and dehydrated with graded alcohol washes (95%–100%) for 2 minutes and xylene 3 times for 1 minute. Slides from both rates at each time point were stained simultaneously, with positive control tissue (rat kidney for FGF-2; rat aorta for CD31, VEGF, and PDGF-AA), and a negative control (rat femur) that used 1.5% blocking serum instead of primary antibody.

Staining in the distraction gap was reviewed and characterized using an Eclipse E800 polarized light microscope (Nikon, Melville, NY). Digital photomicrographs were taken and processed with the Spot RT Slider camera and software (Diagnostic Instruments, Inc., Sterling Heights, MI). Positive immunostaining was typified by the appearance of light to dark brown intracellular coloring and qualitatively graded as strong, moderate, and weak. To be graded as strong, 50% or more of all cells positive/high-power field (40×) had to stain dark brown. A moderate grade was 25% to 50% cells positive/high-power field (40×) staining light to dark brown. Weak grading was 25% or less cells positive/high power field (40×) staining light brown, and an absent grade demonstrated no cellular staining. All specimens were compared with positive and negative control tissues processed with identical immunohistochemistry protocols. Sections of treated bone processed without primary antibody were used as negative controls.

Histological appearance and antibody staining were characterized in 3 different zones of the regenerate within the distraction gap: (1) the central fibrous zone, located in the middle of the distraction zone, consisting of granulation tissue and developing blood vessels; (2) the primary mineralization front, adjacent to the central fibrous zone, consisting of mesenchymal cells and osteoblasts in vascularized fibrous tissue with small islands of woven bone aligned parallel to the distraction force; and (3) The peripheral new bone zone, between the primary mineralization front and the preexisting cortical and medullary bone, consisting of mineralized woven and lamellar bone.8
RESULTS

The bones from 52 (0.5 mm/d, n = 26; 1.5 mm/d, n = 26) animals were available at the end of the experiment; 4 animals from each group were excluded because of technical problems at the time of surgery or because they had deep infections at the time of euthanasia. One animal was lost from each killing time point except day 21 in the slow-distraction group, whereas 1 animal was lost from each killing time point except day 12 in the fast-distraction group. All 52 remaining specimens were reviewed and graded for growth factor expression (Table 1).

Histological Evaluation

In the slow-distraction animals, fibroblasts and macrophages filled the distraction gap and central fibrous zone on postoperative day 8 (0.5 mm of distraction). Subperiosteal bone expansion and osteoblast proliferation were evident adjacent to the distraction gap (Fig. 1A). On day 12, there was continued periosteal cell proliferation and the formation of new bone in the distraction gap and central fibrous zone (Fig. 1B). The central fibrous zone contained granulation tissue and numerous longitudinally aligned blood vessels. On day 14, subperiosteal bone and vascularized granulation tissue bridged the distraction gap, and a primary mineralization front had begun to develop from the cut bone ends. By day 21, the central fibrous zone was nearly replaced by woven bone. The mineralization front and regenerate bone were aligned parallel to the distraction force. Longitudinally arranged fibrous tissue and subperiosteal woven bone nearly bridged the distraction gap (Fig. 1C).

On day 8 (1.5-mm distraction), the distraction gap in the fast-distraction specimens contained loosely organized fibroblast and macrophage-laden granulation tissue (Fig. 1D) and a small amount of periosteal and subperiosteal bone proliferation. By day 12, the bones had been lengthened the full 7.0 mm. In all 6 animals in this group, the distraction gap contained a large, central cyst that was surrounded by granulation tissue and few areas of regenerating bone (Fig. 1E). The cyst essentially filled the distraction gap, although there was some periosteal new bone formation and a moderate amount of cartilage peripherally. On days 14 and 21, there were remnants of cysts in the distraction gap and increasing amounts of longitudinally arranged fibrous tissue. However, a paucity of regenerate woven bone was observed in the distraction zone except at the corticotomy-periosteal interface (Fig. 1F).

Immunohistochemistry of PDGF-AA

In the slow-distraction specimens, there was strong staining for PDGF-AA in osteoblasts in the subperiosteal bone adjacent to the distraction gap and central fibrous zone on day 8. Platelets and fibroblasts stained strongly in the central fibrous zone on day 8, changing to moderate by day 12. Osteoblasts in the subperiosteal bone adjacent to the distraction gap and between the central fibrous zone and intramedullary bone stained only weakly for PDGF-AA on day 12 (Fig. 2A). After day 12, weak staining was evident in endothelial cells and occasional fibroblasts at the slow rate (Fig. 3A). In the fast-distraction specimens, there was virtually no staining outside of the central fibrous zone and only weak staining in platelets and fibroblasts on day 8. In contrast to slow distraction, there was moderate to weak expression of PDGF-AA in fibroblasts and platelets in the central fibrous zone but no peripheral staining (Fig. 2B). There was no PDGF-AA staining in the fast-distraction specimens from animals killed on days 14 and 21 (Fig. 3B).

Immunohistochemistry of FGF-2

There was moderate staining of FGF-2 in the peristeum, central fibrous zone fibroblasts, and osteoblasts in the subperiosteal bone adjacent to the distraction gap at day 8 in the slow-distraction group. By day 12, there was strong expression in the fibroblasts and mesenchymal cells in the central fibrous zone and developing primary mineralization front, and in the osteoblasts in the primary mineralization front and intramedullary bone (Fig. 2C). Fibroblast growth factor 2 staining intensity decreased in the mesenchymal cells and osteoblasts in the mineralization front and peripheral new bone by days 14 and 21 (Fig. 3C). There was a similar pattern of staining through day 12 in the fast-distraction animals, although fewer fibroblasts, mesenchymal cells, and osteoblasts in the periosteum, subperiosteal bone, distraction gap, and distraction zone stained positively (Fig. 2D). There was no FGF-2 staining on days 14 and 21 in the fast-distraction animals (Fig. 3D).

Immunohistochemistry of VEGF

Fibroblasts, mesenchymal cells, and small blood vessels located in the periosteum, adjacent soft tissue, and the central fibrous zone stained moderately intensely for VEGF on day 8 in the slow-distraction group. Osteoblasts in the subperiosteal bone adjacent to the distraction gap and in the intramedullary bone adjacent to the central fibrous zone exhibited more moderate staining. There was strong staining of VEGF in fibroblasts, mesenchymal cells, and newly

![Table 1. Specimens Graded for Growth Factor Expression](https://example.com/table1.png)

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Semiquantitative grading of cytokine staining throughout the regenerate, including the central fibrous zone, primary mineralization front, and peripheral new bone zone. +++ indicates strong (dark brown, ≥50% cellular staining); ++, moderate (light to dark brown, 25%-50% cellular staining); +, weak (light brown, ≤25% cellular staining); --, absent.

TABLE 1. Specimens Graded for Growth Factor Expression
formed blood vessels in the central fibrous zone and mineralization front, and in osteoblasts in the primary mineralization front and intramedullary bone by days 10 and 12 (Fig. 2E). On days 14 and 21, the mesenchymal cells and osteoblasts in the mineralization front and peripheral new bone stained only weakly for VEGF in the slow-distraction animals (Fig. 3E). The staining patterns in the slow-distraction specimens were similar to those in the
fast-distraction specimens through day 12, although fewer fibroblasts, mesenchymal cells, and osteoblasts in all zones of the distraction gap stained positive compared with slow distraction (Fig. 2F). There was no qualitatively measured antibody binding VEGF staining in the fast-distraction specimens on days 14 and 21 (Fig. 3F).

FIGURE 2. Immunostaining pattern of longitudinal section of distraction zone on day 12: Left column slow distraction (0.5 mm/d), right column fast distraction (1.5 mm/d). A, Moderate PDGF-AA staining in osteoblasts and mesenchymal cells (arrows) between central fibrous zone and intramedullary bone (20×). B, Weak PDGF-AA staining in platelets and mesenchymal cells (arrow) bordering central fibrous zone and intramedullary bone (20×). C, Strong FGF-2 staining in mesenchymal cells, osteoblasts (arrow), and chondrocytes (40×). D, Moderate FGF-2 staining in osteoblasts (solid arrow) (40×). E, Strong VEGF staining in mesenchymal cells (open arrow) and osteoblasts (solid arrow) (20×). F, Moderate VEGF staining present in mesenchymal cells (open arrow) and osteoblasts (solid arrow) (20×). G, Strong CD31 staining in developing central fibrous blood vessels (arrows) (40×). H, Weak CD31 staining present in endothelial cells (arrow) (40×).
Immunohistochemistry of CD31

Small blood vessels in the soft tissues adjacent to the periosteum and within the fibrous granulation tissue in the distraction gap stained strongly for CD31 on day 8 of slow distraction. The strong staining continued through day 12 in soft tissue blood vessels and developing vessels in the central fibrous granulation tissue.
In the present study, whereas FGF-2 is both osteogenic and angiogenic. PDGF-AA, FGF-2, and VEGF had similar spatial patterns of staining that were most apparent at the early time points, days 8 to 12, at both rates of distraction. However, distraction of 1.5 mm/d led to decreased cellular staining in fibroblasts, mesenchymal cells, osteoblasts, and endothelial cells compared with 0.5 mm/d. FGF-2 and VEGF are highly expressed in fibroblasts, osteoblasts, and osteoprogenitor cells within the distraction zone as observed by Yeung et al., Hu et al., and Pacicca et al. However, expression at a distraction rate of 1.5 mm/d was weak to moderate through day 12, becoming absent thereafter. CD31 stained strongly in the distraction zone at 0.5 mm/d of distraction, which is consistent with what was observed previously by Li in a rat distraction model. Li found less CD31 staining at 1.3 compared with 0.5 mm/d in the distraction zone within endothelial cells, which mirror our results.

The principal limitation of this study was the difference in distraction stimulus in the 2 groups at the late time points. After postoperative day 12, the fast-distraction group was no longer being distracted, unlike the slow-distracted specimens that had a distraction force applied until day 21. Distraction osteogenesis has been shown to provide a stimulus for cellular proliferation and differentiation in the distraction zone. Mitotic activity and growth factor expression increase with callus distraction, and distraction enhances the orientation of callus formation and cellular alignment. In our rapidly distracted animals, distraction ceased at day 12, at which point, we found cartilage islands and cysts within the distraction zone. In contrast, the slowly distracted group had a consistent distraction force applied, promoting continued cellular growth factor expression, proliferation, and differentiation.

Bone may heal slowly or not at all after distraction osteogenesis, even in ideal situations, and fast distraction rates (>1 mm/d) have been shown empirically and experimentally to be unsafe. Poor bone formation is only one of the problems caused by rapid distraction. Rapid distraction also adversely affects nerve, muscle, and blood vessels. This suggests that bony nonunion may be due in part to disruption of angiogenesis-related cytokine signaling. It also suggests that adding exogenous cytokines may provide some therapeutic benefit for bone healing. The data in slow distraction models are mixed. Although VEGF alone does not seem to work, FGF-2 has been shown to increase regenerate bone mineral content in rabbits, and PDGF-BB clearly increases regenerate new bone formation in a rat model of distraction osteogenesis (unpublished data). It remains to be seen whether FGF-2, PDGF-BB, or other as yet untried growth factors will be capable of resuming failed healing due to rapid distraction.

In summary, this study has demonstrated rapid distraction results in decreased angiogenic growth factor expression and decreased new vessel formation as evidenced by decreased CD31 staining. Our results suggest the inhibition of angiogenesis.

**DISCUSSION**

Normal osseous repair of a bone defect requires an adequate blood supply and a cascade of molecular signals that coordinate the reparative process through a sequence of cellular differentiation and organization. Previous works had suggested that rapid lengthening may lead to disruption of the periosteum and the vascular supply. In the present study, we examined whether rapid lengthening might influence angiogenesis by altering the expression of FGF-2, VEGF, and PDGF-AA. In fact, starting on the first day of lengthening (postoperative day 8), we found that rapid distraction led to decreased cellular staining of FGF-2, VEGF, and PDGF-AA, and decreased angiogenesis, as evidenced by decreased CD31 staining in the distraction zone. We also found that at our fast rate of distraction (1.5 mm/d), no regenerate bone was formed in the distraction zone at any time point. This suggests that rapid distraction disrupts the normal angiogenic signaling sequence.

Our study focused on the expression of FGF-2, VEGF, and PDGF-AA because we were primarily interested in the effect of lengthening rate on angiogenesis and the osteogenic cascade. Successful osteogenesis requires early new blood vessel formation and mesenchymal cell migration and proliferation in the distraction gap. Vascular endothelial growth factor and PDGF-AA are potent stimulators of angiogenesis that have been identified during bone healing, whereas FGF-2 is both osteogenic and angiogenic. Certainly, there are other growth factors that are involved in bone healing during distraction osteogenesis such as the bone morphogenetic proteins and other critical components of the cytokine signaling pathways (e.g., receptors and transcription factors). However, the evaluation of these was beyond the scope of the present study.

In our model, we found palisades of new bone aligned parallel to the direction of distraction on postoperative day 21 (14 days of distraction) in the slow-distraction (0.5 mm/d) animals but no new bone at any time point in the fast distraction animals (1.5 mm/d). This is consistent with the findings of Aronson et al., who observed more mineralized bone at 0.5 mm/d of distraction compared with distraction of 2.0 mm/d, and Choi et al., who found distraction of 0.5 mm/d fostered callus formation and bony union via intramembranous ossification but fibrous tissue interposition, cartilage formation, and nonunion when the distraction rate was increased to more than 1.0 mm/d. In addition, in our study, by postoperative day 10, rapid distraction produced large, hemorrhagic cysts that filled the distraction zone and cartilage islands at the distraction gap adjacent to bridging sub-periosteal bone. Cystic degeneration and cartilage island formation were previously reported to be a consequence of rapid distraction, leading to poor regenerate bone or nonunion.

Bone may heal slowly or not at all after distraction osteogenesis, even in ideal situations, and fast distraction rates (>1 mm/d) have been shown empirically and experimentally to be unsafe. Poor bone formation is only one of the problems caused by rapid distraction. Rapid distraction also adversely affects nerve, muscle, and blood vessels. This suggests that bony nonunion may be due in part to disruption of angiogenesis-related cytokine signaling. It also suggests that adding exogenous cytokines may provide some therapeutic benefit for bone healing. The data in slow distraction models are mixed. Although VEGF alone does not seem to work, FGF-2 has been shown to increase regenerate bone mineral content in rabbits, and PDGF-BB clearly increases regenerate new bone formation in a rat model of distraction osteogenesis (unpublished data). It remains to be seen whether FGF-2, PDGF-BB, or other as yet untried growth factors will be capable of rescuing failed healing due to rapid distraction.

In summary, this study has demonstrated rapid distraction results in decreased angiogenic growth factor expression and decreased new vessel formation as evidenced by decreased CD31 staining. Our results suggest the inhibition of angiogenesis.
of new vessel formation and subsequent nonunion seen with rapid distraction are due, at least in part, to early disruption in angiogenic cytokine signaling, which coordinates the osteogenic cascade within the distraction gap. If that is the case, it may be possible to support bone healing with the appropriately timed application of exogenous growth factors. Of course, other strategies will be required to resolve the problems caused by fast distraction of the soft tissues.

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REFERENCES