Increased Distraction Rates Influence Precursor Tissue Composition Without Affecting Bone Regeneration*

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ABSTRACT

The effect of increased distraction rate on bony tissue differentiation was studied using a paired bilateral model of rat femur lengthening. After a 6-day latency period, one randomly selected femur for each rat was distracted at 0.5 mm/day (normal rate) for 12 days, and the contralateral femur was distracted at 1.5 mm/day (increased rate) for 4 days. Femoral lengthening for each side was 6.0 mm, leaving the increased rate leg with an extra 8 days of consolidation compared with the normal rate limb. Group I rats (n = 9) were killed at day 18 postsurgery and analyzed for cartilage tissue composition and distribution. Group II rats (n = 7) were killed on day 36 postsurgery and analyzed by three-dimensional microcomputed tomography (MCT) for changes in new bone volume. Digital color analysis of slides stained with type II collagen antibody showed increases in cartilaginous tissue formation on the increased rate side (1.51 mm² vs. 0.83 mm²; p = 0.10). No differences in new bone volume were detected between increased rate limbs and their contralateral controls (46.13 mm³ vs. 42.69 mm³; p = 0.63). These findings suggest that intermediate distraction rates may influence precursor tissue composition without affecting the final amount of new bone formed. Because damage to the tissue was not detected at either time point, these changes in chondrogenesis may reflect sensitivity of the pluripotential gap tissue to tension accumulation during lengthening. Future work with this in vivo model is focused on improving our understanding of the mechanisms behind this strain sensitivity. (J Bone Miner Res 2000;15:982–989)

Key words: distraction osteogenesis, chondrogenesis, bone regeneration, strain, damage

INTRODUCTION

DISTRACTION OSTEOGENESIS (DO) is an increasingly popular technique used to stimulate new bone formation to treat orthopedic disorders resulting from bony defects and deficits. Distraction can be performed on a variety of bones, from the femur to the mandible, and lengthenings of greater than 10 cm have been reported (J.A. Goulet, personal communication, 1996). (1–3) One principle generally accepted as critical to the technique’s success is the separation of the two bony ends at a slow, controlled rate of about 1 mm/day. Ilizarov, who pioneered most clinical applications of DO, repeatedly stressed the importance of “slow, steady traction” for successful bone regeneration. (1,4,5) However, faster distraction rates might reduce the amount of time required for lengthening and subsequent consolidation. Shorter treatment times also might decrease the incidence of pin site infections and other complications. (2,3) This has prompted orthopedic surgeons to ask whether distraction rates might be increased to shorten treatment time.

Most of the evidence supporting a rate of 1 mm/day has come from clinical outcome studies. Often, these results are simply observations that this rate was successful, and possible confounding factors are not accounted for in these reports. (1,6–8) The failure of the Wagner technique to bridge

spontaneously the gap with bone after distracting the two bony ends as quickly as the soft tissues will allow, has been well documented.\(^9\) Ilizarov, in his classic canine experiments, showed that increasing the distraction rate from 1.0 to 2.0 mm/day was detrimental to gap tissue ossification.\(^5\) However, these results were largely qualitative, and sample sizes within the many different groups were small.

Several investigators have studied how tension accumulates within the distraction gap during lengthening.\(^{7,8,10}\) Rapid tension accumulation has been postulated to contribute to decreased healing at higher distraction rates. This is supported by the clinical observation that joint contractures, a common complication of limb lengthening, are ameliorated by stopping distractions for a few days.\(^3\) Recent work by our group has quantified the viscoelastic nature of gap tissue to predict tension occurring during the distraction process.\(^{11}\) However, cases also have been reported in which distraction rates had to be increased in order to avoid premature consolidation. This is especially true for younger patients and for lengthenings over intramedullary nails (J.A. Goulet, personal communication, 1996).\(^12\)

This body of evidence raises several important questions. Do modest increases in distraction rate prove detrimental to bone regeneration? Do they affect precursor tissue composition within the gap? If so, what are the mechanisms responsible for these changes? Answers to these questions would likely improve our understanding of how physical forces and strains influence bone repair and regeneration at both the tissue and the organ levels. Answers to these questions also might allow surgeons to tailor distraction protocols to optimize patient care. The objectives of this study were to determine whether increases in distraction rate (1) affect new bone volume and (2) alter the amount or distribution of cartilaginous tissue within the distraction gap. This article presents paired data from a bilateral rat femoral distraction model. Rats were killed at both an early and a late time point to study the effects of increased distraction rate on both the soft and the mineralized tissues that contribute to bony healing during limb lengthening.

**MATERIALS AND METHODS**

**Surgery, distraction protocol, and euthanasia**

Bilateral lengthening of femora in male Sprague-Dawley rats, 1 year in age and 500 g in weight, was performed. All procedures were conducted under general anesthesia and sterile conditions. Using a custom drill guide, four threaded Steinmann pins, 0.067 in. in diameter, were placed in the anterolateral aspect of both femora after percutaneous predrilling. A custom, monolateral external distraction device was then placed over the pins and locked into place (Fig. 1). The diaphysis of the femur was then exposed via a small anterolateral incision and blunt dissection through the overlying tissues. After elevation and protection of the periosteum, the femur was transected using an oscillating saw with saline irrigation. The wound site was cleaned of bone debris, and the distraction gap was closed, bringing the two cortical bony ends into contact. Subcutaneous tissue and skin were closed with 3.0 vicryl suture and staples, respectively. All animals were fully weight bearing less than 1 h after surgery and were given chow and water ad libitum until the time of death. Pin sites were cleaned daily with antibiotic solution. Weekly radiographs were taken to monitor animals for the presence of fractures, pin site infections, or malreduction of the bony ends (Fig. 2). All rats in this study followed the same distraction protocol. After a 6-day latency period, one randomly chosen femur was distracted 0.50 mm/day (0.25 mm, two times daily) for 12 days. The contralateral femur was distracted at three times this rate (0.75 mm, two times daily) for 4 days. Distraction for both legs began on the same day after the 6-day latency period. The total femoral lengthening was 6.0 mm on both sides and was completed on day 10 postsurgery.
for the increased rate leg and day 18 postsurgery on the contralateral limb. Rats were divided into two groups. Group I rats \((n = 9)\) were killed on day 18, immediately after lengthening was finished, to study soft tissue development within the distraction callus. Group II rats \((n = 7)\) were killed on day 36 to determine mineralized bone volumes within the gap. Rats were killed by carbon monoxide asphyxiation. Limbs were removed by disarticulation and stripped of muscles and other superficial tissues. Distraction zone tissue was harvested with the fixators and neighboring cortical bone intact to ensure it was not damaged during processing. Gap tissue was immediately fixed in cold 10% neutral buffered formalin (NBF) for 48 h. Afterward, samples were placed in cold 70% ethanol while awaiting further analysis. The University Committee on Use and Care of Animals approved all procedures at The University of Michigan, in accordance with National Institutes of Health (NIH) guidelines.

**Microcomputed tomography**

While in ethanol, distraction zones from Group II rats (day 36) were scanned by three-dimensional microcomputed tomography (MCT) at a resolution of 50 \(\mu m/voxel\) (Fig. 3).\(^{13}\) MCT images were reconstructed at a byte range such that a voxel intensity of 255 corresponded to fully mineralized cortical bone. From these images, histograms of voxel count versus voxel intensity within the distraction gap were constructed. Voxel counts were obtained in two regions of interest: (1) gap only tissue (BVGap), taken as tissue within the gap bounded by the radial dimensions of the original cortex; (2) BVFull, an extended count, which included the entire distraction callus. This technique has previously been used to determine new bone volumes in a rabbit model of DO.\(^{12,14}\) Information regarding soft tissues within the gap cannot be obtained from MCT images. Distraction zones from group I rats (day 18) possessed little or no mineralized bone within the gap and, therefore, were not scanned.

**Histology**

Group I rats were decalcified and embedded in paraffin using standard histological techniques. Seven-micrometer-thin longitudinal sections were taken through the distraction gap and stained with toluidine blue (1%) and safranin-O (1.0%)/fast green (0.02%) for qualitative analysis under light microscopy. A second set of slides were stained with type II antibody (II-II6B3)\(^{15}\) for detection of cartilage within the distraction gap. Briefly, slides were hydrated and incubated with hyaluronidase (5 mg/ml) for 1 h at 37°C. Methanol/peroxidase quenching was performed to diminish endogenous peroxidase activity. After blocking with 10% bovine serum albumin (BSA) solution, slides were incubated with a 1:300 dilution of the antibody for 2 h at 37°C. Primary antibody was detected using a commercial biotinylated secondary antibody and chromagen kit (Zymed, South San Francisco, CA, U.S.A.). No counterstaining was performed to enhance contrast between regions of positive and negative staining. Rabbit and rat growth plates were used as positive controls; slides stained with no primary antibody served as negative controls.

Group II rats were dehydrated and embedded undecalciﬁed in polymethyl methacrylate (PMMA) (after MCT scanning) in a specific known orientation. Following a previously described protocol,\(^{16}\) embedded blocks were thicknessed at 1000 \(\mu m\) using a diamond-blade saw (EXAKT, Oklahoma City, OK, U.S.A.). Thick sections were mounted on radiolucent plastic, polished, and surface-stained with toluidine blue for qualitative histological analyses (Fig. 4), using an established histological protocol.\(^{17}\)

**Digital color analysis**

Digital color analysis was performed to quantify areas of cartilaginous tissue on antibody-stained slides from group I rats. Color images of two midsagittal slides from each distraction zone in each rat were captured (Javelin, Torrance, CA, U.S.A.; NIH Image, Bethesda, MD, U.S.A.).
Images were thresholded to separate positive-staining cartilaginous pixels from other pixels and counted (MATLAB, Mountain View, CA, U.S.A.) to determine the area of cartilaginous tissue within the gap. Image resolutions ranged from 20 to 30 μm/pixel (Fig. 5). Paired distraction zones (and their respective slides) were obtained, processed, stained, and imaged simultaneously to increase the consistency of paired analyses. All slides were examined carefully to ensure that only tissue that possessed the morphological features of cartilage was counted in the color analysis.

Statistical analysis

Paired t-tests were performed to detect differences in outcome variables between paired rats in groups I and II (SYSTAT, Evanston, IL, U.S.A.). Results were considered significant at a level of \( p \leq 0.05 \).

RESULTS

Surgical results

Two rats were lost during surgery to intraoperative fractures of the femur. Otherwise, rats tolerated surgery and the increased rate of distraction well. A predisposition to pin site infections was observed for some of the group II rats because of their longer time of study. However, none of these infections caused pin loosening or compromised the tissue within the distraction gap. Rats resumed normal cage activity less than 1 h after surgery; it should be stressed that at no time did animals appear to favor one limb over another in this bilateral model.

MCT and new bone volume

Three-dimensional MCT images indicated that bone regeneration was most prevalent in periosteal locations (Fig. 3). Often, this new periosteal bone appeared compact and lacked trabecular features. Histograms obtained from MCT images exhibited a bimodal distribution that separated mineralized and nonmineralized voxels within the distraction gap tissue. This separation between the two voxel populations was present in histograms constructed for all animals killed at 36 days. Both the BVGap and the BVFull measures of new bone volume were increased slightly in rat femora distracted at the higher rate (Fig. 6). However, these differences were small and not statistically significant (BVGap, 46.13 mm³ [1.5 mm/day] vs. 42.69 mm³ [0.5 mm/day], \( p = 0.63 \); BVFull, 82.21 mm³ [1.5 mm/day] vs. 75.05 mm³ [0.5 mm/day], \( p = 0.53 \)).

Histology

Light microscopy analysis of sections from groups I and II stained with toluidine blue confirmed that alignment of the two bony ends was maintained throughout distraction (Fig. 2). Only small amounts of differentiated tissue were present in the gap center at 18 days, particularly near the marrow at proximal and distal ends. Abundant fibrous tissue was seen outside the gap in periosteal regions, giving the appearance of a large fracture callus. More tissue proliferation generally was seen on the side opposite the distractor. Cartilage was seen in all samples in varying amounts, indicating that bone was likely forming by both endochondral and intramembranous mechanisms. Tissue orientation along the applied tension vector of the bone was evident, especially in samples distracted at the increased rate. In agreement with the MCT data from group II rats, new bone formation activity at 36 days was focused in periosteal regions of the distraction gap (Fig. 4). Some of this bone appeared lamellar, mature, and lacked any trabecular features. In contrast, regions in the center of the distraction gap often showed little evidence of mineralized bone formation. Rigorous morphological analyses of trabecular bone archi-
tecture could not be performed because an inadequate number of fields containing trabecular bone were present.

Digital color analysis

Digital color analysis of group I rats showed differences in the amount of cartilage between femora distracted at the two different rates. Analyses of slides stained with type II antibody indicated a nearly 2-fold increase in cartilaginous tissue area (1.51 mm² vs. 0.83 mm², p = 0.10; Fig. 5). This trend did not achieve statistical significance.

Discussion

Optimal distraction rates for leg lengthening have been defined largely in the clinical arena to lie somewhere between two undesirable extremes. At low distraction rates, premature consolidation occurs, and the lengthening goal is not achieved. On the other hand, at faster rates, the distraction gap and surrounding tissues might be damaged, hindering new bone formation. However, the true effect of distraction rate on bone regeneration is not well understood. In this study, increased rates of lengthening were implemented in a bilateral rat model of distraction to investigate their effect on bone regeneration. In addition, gap tissue in the rat model before mineralization was studied to detect changes in cartilaginous tissue differentiation. In this fashion, the effects of increased stretch (and associated increases in tension levels) on bone regeneration at two different time points could be studied and correlated with one another.

Higher distraction rates were associated with increases in cartilage formation at 18 days postsurgery. Staining for type II collagen was more reliable than the safranin-O technique; however, both methods showed the observed increase in chondrogenesis. The presence of cartilage was always associated with bone formation activity and usually was in a periosteal location. Thus, the majority of tissue differentiation at this early stage of bone repair appeared to be occurring in periosteal regions of the gap, in agreement with observations in other fracture and distraction models. The 3-fold increase in distraction rate had no effect on new bone volumes at 36 days postsurgery; thus femora distracted at both rates appeared to make bone at similar rates.

It should be stressed that these experiments were not carried out to the time point at which bony bridging of the gap occurred. Two rats killed at 64 days postoperatively displayed nearly complete bridging of the gap (unpublished data, Richards and Goldstein, 1997), indicating that the model is successful and does not induce pseudarthrosis formation. It is not likely that differences in new bone volume between the normal and increased rate legs would appear between the 36-day and 64-day time points, but this cannot be ruled out based on the data presented here.

In this study, the use of an oscillating saw to produce the osteotomy, rather than a more clinically recognized corticotomy, was chosen to produce a uniform distraction gap geometry and to avoid the oblique fractures often seen in corticotomies. Some authors claim that corticotomies provide a faster healing response by maintaining the medullary blood vessels intact, in comparison with an osteotomy made all the way through the marrow cavity. Others see no evident difference in healing patterns between the two. (19) Frierson advises against the use of an oscillating saw for osteotomies, because of an observed delayed consolidation in dogs. (20) Despite the use of copious saline irrigation throughout the osteotomy in the present study, it is possible that the temperature increased at the osteotomy site during the procedure. This could, in theory, cause thermal damage, which could, in turn, lead to cartilage formation within the distraction gap. However, it is unlikely that this mechanism is responsible for the observed increase in cartilage seen in the increased rate leg of the animal compared with the normal rate leg, because the osteotomy protocol was the same regardless of distraction rate.

Various mechanisms might be advanced to account for the increases in cartilage formation, many of which center around damage. Increased distraction rates might lead to higher peak stresses and strains immediately after distraction; likewise, more rapid tension accumulation during the full course of lengthening may occur at higher distraction rates. Such patterns of tension accumulation have been documented in both clinical studies and work describing the viscoelastic properties of gap tissue. (8,11) These altered physical forces and strains then likely exert their influence on important biological processes. Damage to newly forming blood vessels, the peristome, and/or scaffolding elements within the distraction gap might decrease vascularity or oxygen tension to the tissues, rendering them more amenable to cartilage differentiation than other tissue types. (23) Existing blood vessels may become damaged at high distraction rates, further reducing the overall blood supply to the wound. (23) However, it cannot be ruled out that the tissue may be stretched at a rate that simply exceeds the capacity to generate blood vessels, causing a deficit of blood supply within the gap. Li and coworkers have found that high rates of distraction inhibit or reduce angiogenesis within a rabbit model of DO. (24) At rates of 0.7 mm/day and 1.3 mm/day, newly formed bone was always associated with blood vessels. Additionally, at rates above 0.7 mm/day, cartilage islands were found within the tissue and were not associated with angiogenic markers. A similar phenomenon could account for the higher cartilage formation seen in the present experiment on the legs distracted at the increased rate of 1.5 mm/day.

Although damage to the tissue within the distraction gap may have influenced the resulting tissue type, it also is possible that the observed increase in chondrogenesis was the result of local strain influences on fundamental processes of differentiation from pluripotent mesenchymal tissue. Pauwels, Carter, and others have hypothesized that local strain environment influences cellular events, including differentiation and gene expression. (25–28) Specifically, they have argued that high distortional strains favor fibrous tissue formation, whereas more “quiescent” strain regions favor the formation of cartilage. Evidence to support the existence of such a mechanism comes from the cellular biology literature. (29–31) Experiments have shown that chondrocytes grown on adherent media “dedifferentiate” and begin to express type I collagen as they assume a more
elliptical shape. Resuspension of these cells in media allowed the chondrocytes to reassume their spherical shape, at which time they again expressed type II collagen.

Thus, it has been argued that strains with a high distritional character might discourage chondrogenesis. On a superficial level, these views are not consistent with the findings in this study. As stretches are imposed along a single axis, it might be expected that increased distraction rates would lead to increased distortion of the tissue, especially in the direction of applied step elongations. Such a theory would argue for increased fibrous tissue formation in such a mechanical environment. However, in this study, increased distraction rates appeared to favor chondrogenesis and not fibrous tissue formation. It should be emphasized, though, that information regarding a local strain environment was not rigorously established for changes in distraction rate in this animal model. This would require nonlinear, three-dimensional, viscoelastic finite element analyses, beyond the scope of the present study.

Strain-related inhibition of chondrogenesis has been postulated to occur in another rat distraction model, in which the formation of cartilage within a distraction gap was only observed after the distraction protocol was halted.\(^{(32)}\) In this study, rat femora were distracted 0.165 mm every 12 h. At this slower distraction rate, the gap was bridged completely by bone when the final length of 7 mm was obtained, and at this point, no cartilage was observed. After 21 days of consolidation, some parts of the distraction gap showed columns of chondrocytes and cartilage, suggesting endochondral bone formation. The lack of cartilage formation during distraction was attributed to the generation of tensile distraction forces. However, it is also possible that this low distraction rate (0.33 mm/day) reduced tension accumulation and potential damage within the gap. This mechanical environment may then have been more permissive of fibrous tissue and new bone formation.

Although increased distraction rates did influence cartilaginous tissue production, they did not have any effect on new bone volume at 36 days postsurgery in this animal model. No differences in bone volume were detected between the normal and increased distraction rate femora at this time point. Thus, new bone volume was unaffected by any potential prior differences in cartilaginous tissue formation. This finding is different from those of Aronson et al., who qualitatively described improved mineralized bone formation with tibial lengthenings of 0.5 mm/day, compared with 2.0 mm/day.\(^{(33)}\) There are several important differences between the current study and that of Aronson et al. that might explain this discrepancy. Their model was of rat tibial lengthening; because the femur has a better blood supply than the tibia, this could impact the results here. Studies by Aronson lengthened only one limb in each animal and used a ring fixator; clearly, this could create very different strain environments within the developing gap tissue. Furthermore, the described changes in new bone formation were not quantified, and their increased rate was one-third faster than that used in our protocol.\(^{(33)}\)

Nonrecoverable tissue damage has been shown to exist in high distraction rate models, in which necrotic tissue and cysts remained within the distracted tissue after 4 weeks of consolidation.\(^{(23)}\) This could suggest that increases above a certain distraction rate result in excessive and irreparable damage to the tissue. In the current study, increased distraction rates caused greater cartilage formation at an early time point but no change in total new bone volume 18 days later. Furthermore, no evidence of necrosis or cysts was seen in any of the increased rate distraction gap tissue samples at 18 days or 36 days. Because of the bilateral nature of our animal model, the increased rate limb had an extra 8 days of consolidation compared with the normal rate limb. Although the higher distraction rate initially may have caused damage reflected by the increased cartilage levels, it is possible that the increased time of consolidation allowed more healing to occur in the high rate limb. Increased endochondral bone formation may have contributed to the levels of bone found at the final time point. In addition, our distraction rates were higher than the rates of Jazrawi et al. (1.3 mm/day), who saw no cartilage formation during the distraction, and lower than the rates of Aronson (2.0 mm/day) and Li (2.7 mm/day).\(^{(32,33)}\) Our observations support the currently held view that an intermediate distraction rate is optimal. By increasing distraction rate to a value that may increase cartilage formation but not cause permanent tissue damage nor hinder new bone formation, the final desired length might be reached at an earlier time point. However, additional consolidation time may then be required in order to achieve required bony filling of the gap.

This raises an important question in its own right. For more than a decade, scientists and clinicians have argued over the role cartilage plays during bone regeneration during distraction. Some practitioners have claimed that bone formation during distraction should be entirely intramembranous. Any cartilage within the distraction gap was assumed to result from either poor fixator stability (and excessive micromotion) or failure to preserve the blood supply during surgery.\(^{(1,4,5,34)}\) Molecular analyses of distraction gap tissue have supported this viewpoint.\(^{(35,36)}\) However, reports of cartilaginous tissue within the distraction gap have become increasingly common in both ring and monolateral fixator systems.\(^{(18 – 22,37)}\) Thus, although these animal models of distraction provide an excellent means for the study of tissue differentiation in response to a wide variety of stimuli, it is possible that during distraction, the degree of bony healing does not depend on the mechanism of its formation.

From a strictly clinical standpoint, it is important to realize that the highest achievable distraction rate depends not only on the response of the newly forming bony tissue but also on constraints placed on it by surrounding tissues. Numerous reports have documented an increased incidence of joint contractures and angular deformities at higher distraction rates,\(^{(7,10,38)}\) likely because of an inability of surrounding muscles to stretch adequately. Concerns regarding the ability of nerves to adapt to high distraction rates also have been raised.\(^{(39)}\) Thus, the effects of increased distraction rate on differentiation of bony precursor tissue and new bone volumes presented here should be appreciated in this larger clinical context. The results of this study, which support the use of an intermediate distraction rate, are in agreement with current clinical views and practice.
Finally, certain specifics of the animal model merit comment. First, the bone lengthened in this study was the femur. Larger animal models typically have lengthened the tibia, because fixators are more easily placed on the distal leg\(^1\). The femur has a much better blood supply than the tibia, and clinical results support the contention that healing occurs more rapidly and reliably in the femur than in the tibia\(^40\). As mentioned before, this may account for discrepancies between the results presented here and those of Aronson\(^33\). Second, species specificity could contribute to the observed insensitivity of bone formation to increased distraction rate in this model. Rats are very robust animals and their growth plates do not close; therefore, they may respond to the challenge of bone regeneration more vigorously than other animals. Current work with this rat model of DO is underway to address these issues and further explore the mechanisms by which increased distraction rates influence chondrogenesis and bone regeneration.

ACKNOWLEDGMENTS

The authors thank A.R. McDonald, E. Alberg, T. Henne, M.J. Hernandez, V. Bhatia, C. DeBano, J.P. Rouleau, M. Stock, D.C. Kayner, K.A. Sweet, and B. Nolan for helping to make this work possible. Statistical consultations were provided by Prof. M.B. Brown, Department of Biostatistics. Support of this work by The Orthopedic Trauma Association, NIH (AR-20557), The Orthopedic Research and Education Foundation, The Whitaker Foundation, and The Orthopaedic Research Labs, University of Michigan is gratefully acknowledged.

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