INTRODUCTION

Distraction osteogenesis (DO) is a widely used orthopaedic treatment for the correction of limb length discrepancies, congenital deformities, non-unions, and the regeneration of large amounts of bone that have been lost due to trauma. It is one of the most dramatic applications of mechanical stimulation as a means of inducing the regeneration of up to 20% the length of a long bone. While DO procedures are used clinically and the surgical techniques have been refined, the basic mechanisms by which this procedure promotes new bone formation is not well understood. One of the primary descriptive features of DO is that it induces new bone formation through an intramembranous process devoid of extensive amounts of cartilage. However, the most intriguing observation is that the regeneration of abundant amounts of bone is accompanied by robust vascularity of the repair tissue throughout the regenerative process. In order to assess the relationship of bone regeneration during DO to the process of angiogenesis, we have developed a murine model of DO. Using this model, we defined the spatial and temporal expression of the angiogenic and bone morphogenetic signals that drive new bone formation during this process.

METHODS

Surgical Procedure: All procedures were performed under an approved IACUC protocol. BALB/c mice (Charles River Labs, Cambridge, MA) weighing 25 to 35 g were used for these studies. All surgeries were performed under general anesthesia by inhalation of isofluorane and O2. The left leg of the animal was shaved and disinfected with an iodine solution. An anterior longitudinal incision was made over the tibia, and the underlying muscles were retracted with care being taken to minimize damage to the peristeum. A 6 mm distraction device (KLS Martin, Jacksonville, FL), which is used for oral surgical augmentation of alveolar bone, was adapted for use in this procedure. It was attached to the upper portion of the tibia by means of a 0.01 inch ligature wire (3M Unitek, Monrovia, CA) that was wrapped around the tibia and secured with bone cement. The distance between the arms of the device attached to the tibia was usually 3–4 mm. Because the fibula and tibia are fused in mice both were bisected with a transverse osteotomy. The osteotomy was created with the use of a serrated scalpel blade under constant cold saline irrigation and placed symmetrically between the arms of the distractor. Immediately after the osteotomy, both proximal and distal segments were approximated (if needed), and alignment of the device with the tibia was confirmed by x-ray (Gendex Oralix AC, Milan, Italy). The soft tissue was closed with a no.5-0 gut absorbable suture. After surgery, the animals were individually caged and started to bear weight on the operated leg minutes after recovery. The distraction protocol consisted of three phases: 1) latency phase of 7 days duration, 2) active distraction of 10 days duration, and 3) consolidation phase of at least 14 days duration. The rate of distraction was 0.15 mm twice a day (0.3 mm/day). X-rays were taken on the day of surgery, at the end of the latency period (7 days post-surgery), at the middle of the distraction period (11 or 12 days post-surgery), at the end of the active distraction (17 days post-surgery), at the middle of the consolidation period (23 or 24 days post-surgery), and at the end of the consolidation period (31 days post-surgery).

Tissue and Gene Analysis: Histological assessment was made using standard light microscopy. Gene expression levels for end point marker genes defining skeletal tissue differentiation and BMP expression were analyzed by RNAsensitive analysis (RPA) using commercially available template sets per the manufacturer’s recommended protocol (Pharmingen, Inc., San Jose, CA). For the analysis of the differential expression of the multiple metalloproteinase and angiogenesis associated genes, we used a number of specific micro-arrays purchased from SuperArray, Inc. (Bethesda, MD). Arrays were carried out and scanned, and gene expression was normalized to housekeeping genes and controls as per the manufacturer’s instructions. RESULTS

Whole Tissue Analysis: Characterization of both the x-ray assessment and the histological progression of bone repair in this model of distraction osteogenesis demonstrated that the tibia remained aligned throughout the procedure, and expansion of the device over a ten day period was able to produce robust new bone formation during the period of distraction. After the two weeks of consolidation after distraction was completed histological inspection showed a mixture of both lamellar and primary woven bone in the distraction gap.

BMP and ECM Gene Expression: Analysis of extracellular matrix gene expression at the end of the latency period, at two times during the ten day distraction period, and at the end of the consolidation period, showed that new bone formation was induced throughout the period of bone distraction. Interestingly, the expression of SPARC, osteopontin, bone sialoprotein and fibronectin were maximally expressed during the active distraction period. In contrast, osteocalcin and type I collagen, while elevated during the distraction period, showed persistent high levels of expression throughout the consolidation phase. During the period of active distraction both types II and X collagen were expressed at low levels, yet the expression of these genes disappeared completely during the consolidation phase. Examination of the profiles of BMP expression showed similar results with very high levels of expression of BMP2, 3, 4, 5, 6, 7 and 8. These genes were all seen maximally during the period of active distraction and persisted into the consolidation period. It is interesting to note that BMPs 8A, 3 and 2 showed maximal induction during the distraction period.

Angiogenesis and MMP Gene Expression: Of the 96 angiogenesis associated genes that were analyzed with the microarray used for this study, a number of genes were shown to be selectively induced during the distraction period. This included Hif1α (hypoxia induced factor 1A), gelatinase A and B, PEDF, VEGFA, TGFβ−1, endostatin, FGF receptor 3 (FGFR3), angiopoietin 2 and pleiotrophin. We also examined a selected microarray series that is specific for the metalloproteinase (MMP) genes and their inhibitors, as previous studies have shown that the expression of specific metalloproteinases (MMPs) is elevated during periods of active angiogenesis. This particular array contained 20 cDNA fragments from genes encoding the sequences of MMPs and the four specific inhibitors (TIMPs). The results showed that MMP2, MMP8, MMP9, MMP13, and MMP14 were induced as a consequence of the surgical treatment and remained elevated above baseline levels during the tissue repair process. Interestingly, MMP14 appeared to be further elevated during the distraction phase while MMP8 showed a very strong induction during the consolidation phase.

DISCUSSION

We report here the development of a usable and easily adaptable procedure to carry out distraction osteogenesis in mice. We specifically examined the molecular processes related to bone formation during distraction osteogenesis, focusing on both the induction of morphogenetic proteins that promote new bone formation and the expression of angiogenic factors that promote the formation of new blood supply to injured and growing tissues. Our results suggest that the mechanical signals generated during the distraction process induce the expression of select BMPs and angiogenic factors. Multiple BMPs are expressed during DO, and the pattern of their expression is most like late fracture repair. It is of particular interest to note that Hif1α, one of the key transcription factors that is most proximal in the angiogenic regulatory cascade, was induced during the distraction period. These data show the concurrent expression of both angiogenic and bone morphogenetic factors during distraction and suggest complementary and synergistic roles in bone repair and regeneration.


**Department of Trauma and Reconstructive Surgery, University Clinic Hamburg-Eppendorf, Hamburg, Germany

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