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Distraction of Heterotopic Bone Formed with BMP-2 in a Rat Model: The Technique

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Distraction of Heterotopic Bone Formed with BMP-2 in a Rat Model: The Technique

By

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A Thesis submitted in partial satisfaction of
the requirements for the degree of
Master of Science in Orthodontics

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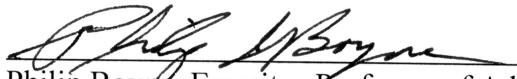

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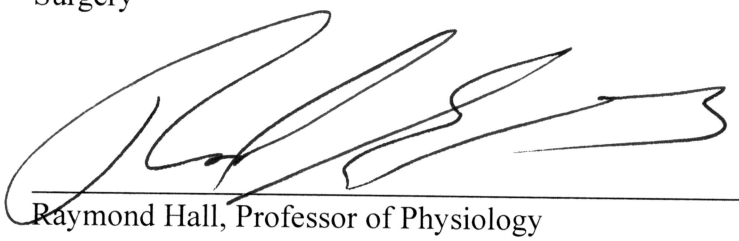

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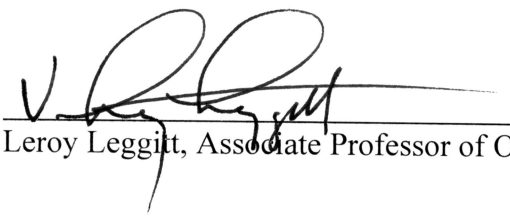

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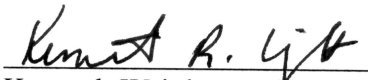

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ABBREVIATIONS

ACS	Absorbable Hemostatic Collagen Sponge
DO	Distraction Osteogenesis
IP	Intraperitoneal
TRAP	Tartrate Resistant Acid Phosphatase
H & E	Hematoxylin and Eosin

ABSTRACT OF THE THESIS

Distraction of Heterotopic Bone Formed with BMP-2 in a Rat Model: The Technique

By

Stacie Dawn Fenderson

Master of Science, Graduate Program in Orthodontics
Loma Linda University, December, 2007
Dr. Philip Boyne, Chairperson

Multiple studies have shown that ectopic bone forms when bone morphogenic proteins (for example BMP-2 and BMP-7) and a collagen matrix are surgically placed in a rat model either orthotopically or heterotopically. Bone begins to form by the 14th day, and is at it maximum calcified state by the 21st day. No study to date has shown what would result from application of force on this ectopic bone. The purpose of this study is to observe and quantify the effect of distraction osteogenesis on the ectopic bone. First, a preliminary procedure was performed by surgically placing a titanium cage into the back of a rat just caudal to the thoracic vertebrae. The anterior corners were sutured to the spinotrapezius muscle for stabilization. The titanium cage containing rhBMP-2 in a collagen carrier was allowed to form bone for 21 days. It was then retrieved and prepared with TRAP stain to observe osteoclast activity. A high osteoclast activity would have indicated that the concentration of BMP-2 might be to high, and the same test would be repeated with a lower concentration. According to Dr. Andresen, various concentrations of BMP-2 had no significant effect on bone formation when used as a bone graft. Once a dose and concentration was established for the optimal bone growth all further studies were done at that concentration. The specimens were evaluated grossly

and histologically for comparison. The dose was given by Medtronics to be 1.4ml of rhBMP-2 per sponge. Between one and one and a half sponges were used per rat. The concentration that formed the most bone was 0.3mg/ml, when compared to 0.1mg/ml. The second phase of the experiment evaluated the effects of expansion by using a distractor like those used in clinical distraction osteogenesis. Instead of removing the cage at 21 days, the forming bone was cut with a 15 blade at day 14 between the two cages attached to the distractor. It was then allowed to "heal" for 1 day, after which expansion began. The rate of expansion was 2/3mm per day for 7 days. A7 day latency period followed expansion before removal and examination of the expanded bone. The bone continued to form beyond 21 days in both types of cages (i.e. distraction and non-expansion). Specimens obtained for evaluation with CT, histology, and grossly.

CHAPTER ONE

INTRODUCTION

In 1965 Marshall R. Urist characterized a protein moiety responsible for the induction of bone growth.[27] This protein material later became known as "bone morphogenic protein". It wasn't until Wozney et al. identified the genetic sequence of bone morphogenic protein, that the various isoforms were developed using recombinant gene technology.[14] The hallmark function of bone morphogenic protein is its ability to induce bone, cartilage, ligament, and tendon formation at both orthotopic and heterotopic sites. Several studies[14,15,17,25,26] have illustrated the potential of bone morphogenic proteins to enhance spinal fusion, repair critical-size defects, accelerate union, and heal articular cartilage. Bone morphogenic proteins have significant promise for potential applications in medical, dental, and veterinary practice for the treatment of cartilage and bone-related diseases.[9] We know that forces, whether tension or pressure, are required to maintain bone; and although many studies have looked at the formation of ectopic bone due to BMP-2, it has yet to be shown what would happen if forces were placed on this ectopic bone. If ectopic bone could remodel and continue to regenerate, then we would have a way to clinically create the size and shape of bone we needed for subsequent transplant procedures without the need for an actual bone grafting. This would decrease the probability of rejection if an allograft were to be used, and in most cases eliminate the need for an autograft. The purpose of this study is to determine if heterotopic bone will regenerate following implantation of BMP-2 and continue to respond to the stimulation of distraction osteogenesis.

CHAPTER TWO

REVIEW OF LITERATURE

Modern technology has decreased the need for organ and tissue donors or even autograft donor sites. A number of studies carried out over the past 20 years have helped to elucidate the roles of BMPs. Although much progress has been made, there are many questions left unanswered. The concept that there is a substance in bone that can induce new bone formation was recognized by Marshall R. Urist in 1965 when he observed that a new ossicle had formed after the implantation of demineralized bone matrix in a muscle pouch of a rat. He termed this phenomenon the bone induction principle and later identified a protein responsible for this effect, to which he gave the name bone morphogenic protein.[27] More than twenty years later, in 1988, Wozney et al. [30] identified the genetic sequence of bone morphogenic protein, which led to the identification of its various isoforms. With this genetic information it is now possible to produce various BMPs with the use of recombinant gene technology.[14]

Bone morphogenic proteins (BMPs) are multifactoral growth factors belonging to the transforming growth factor-beta superfamily. Family members are expressed during limb development, endochondral ossification, early fracture, and cartilage repair. The activity of BMPs was first identified in the 1960s but the proteins responsible for bone induction were unknown until the purification and cloning of human BMPs in the 1980s. Up until 2005, about 15 BMP family members were identified and characterized (More than 20 have been identified since). The signal triggered by BMPs is transduced through serine/threonine kinase receptors. BMPs seem to be involved in the regulation of cell

proliferation, survival, differentiation and apoptosis, but their hallmark is their ability to induce bone, cartilage, ligament, and tendon formation at both heterotopic and orthotopic sites. This suggests that, in the future, they may play a role in the treatment of bone diseases. Several studies[9,14,26] have illustrated the potential of BMPs to enhance spinal fusion, repair critical-size defects, accelerate union, and heal articular cartilage lesions. Difficulties in producing and purifying BMPs from bone tissue have prompted the laboratories to express these proteins in the recombinant form in heterologous systems. [9] In this experiment, we will be looking at rhBMP-2 almost exclusively and the role it plays in bone regeneration.

Bone morphogenic protein 2, or BMP-2, is a protein that induces the formation of bone and cartilage. The gene for BMP-2 is on chromosome 20 in band 20p12.3. Three sets of variations within the BMP-2 gene reportedly triple the risk of developing osteoporosis. Bone morphogenic protein induces ectopic bone formation, and plays an important role in the development of the viscera.[12]

Mandibular distraction osteogenesis is a well-developed clinical modality for the treatment of craniofacial deformities and dental arch discrepancies, in combination with orthodontic treatment. However, other studies have shown that orthodontic tooth movement into the distraction gap caused severe root resorption. The question of whether or not gene expression of osteoclastogenic and osteoclast supporting molecules released by osteoblasts and stromal cells would increase at distraction sites during the consolidation period. The number of osteolclasts was larger in the distraction gap during the early consolidation period than in normal controls, due to an increase of gene expression for osteoclastogenic cytokines in osteoblasts. The conclusion was that

osteoclastogenic and osteoclast activities are stimulated at the distraction sites during the early consolidation period.[29] This research is useful to note clinically, and raises the question whether, if BMP-2 was injected into the consolidation zone, it would consolidate faster enabling the orthodontist to move teeth into that area sooner?

In one of the first published studies with BMPs, the researchers used an aggregate of partially purified bovine bone morphogenic protein (bBMP) and bone matrix insoluble non-collagenous proteins (iNCP). A total of 100 mg of lyophilized BMP/iNCP was implanted, using ultra thin gelatin capsules, in skull trephine defects in adult sheep. In two out of five sheep, the 20 mm skull trephine defects were repaired with bone as early as four weeks after the operation. Eight to 12 weeks after surgery repair was complete in the other three sheep. In the control contralateral trephines, one-third to one half of the defect was incompletely repaired. Neither the BMP nor the BSA control implants induced bone formation in the muscle, while the BMP/iNCP prepared from bovine bone consistently induced regeneration in skull trephine defects; only fibrous tissue and no extraskeletal bone was induced to form in cervical muscle pouches in sheep.[15] This research is important because it shows that a BMP complex is effective even across species, or as a xenograph.

Another study published that same year looked specifically at heterotopic bone formation (HO) as an unsolved problem occurring in response to a variety of conditions including muscle contusions, injuries, surgical procedures and burns. A sensitive method for quantification of HO would be valuable for experimental work and research on bone morphogenic protein (BMP). Roentgenograms, CT scans, MRIs and the like provide a rapid and highly accurate method for determining the amount of HO in a

muscle. Using the mouse thigh pouch as an experimental system, this method demonstrated that quantities of new bone were formed in direct proportion to the amount of BMP/iNCP implanted. This system can be used for measuring HO on roentgenograms or any other image, including computed tomographic (CT) scans, magnetic resonance imaging (MRI), and autoradiograms.[13] It is important to remember that heterotopic bone formation can result simply from a surgical procedure.

A research article published in Germany[28] compared ectopic with orthotopic bone formed in a rat model. The method of this study was to construct a vascularized bone graft using osteoinductive rh-BMP-2, a polylactic acid matrix (OPLA/HY), and a vascularized periosteal flap containing osteoprogenitor cells implanted ectopically in the groin or orthotopically in a femoral defect of a Lewis rat. The presence and density of new bone formation in the groin and femoral defect were evaluated radiologically and histologically at 4 and 8 weeks. Good bone formation in the groin chamber (ectopic) was demonstrated in the periosteal flap + OPLA/HY + BMP group. However, with the presence of a vascularized periosteal flap, more bone formation along the rim of the defect was observed. This study demonstrates that optimal bone formation requires four factors: BMP, a biodegradable matrix, osteoprogenitor cells, and a blood supply. In the future, this technique could potentially be used to reconstruct a bony defect or a nonunion by covering the involved area with a vascularized periosteal flap and a suitable matrix combined with BMP. Alternatively, a vascularized bone graft could be prefabricated at a distant site and then transferred via microsurgery into a defect.[28]

As in the previous study, Stoeger[25] used the technological advances in genetics to test recombinant human bone morphogenic protein-2 (rhBMP-2), along with a

commercial collagen type I carrier, in examining the molecular progression of ectopic bone development in the hind quarter muscles of mice. A gene expression study was performed using mRNA in situ hybridization to compare embryonic cartilage and bone formation with BMP-2 induced ectopic bone formation. It was found that the mRNAs encoding the same molecules, such as Indian hedgehog (IHH), parathyroid hormone (PTH), PTH-related peptide receptor (PPR) and BMPs, that regulate embryonic cartilage and bone development, are expressed during BMP-induced ectopic bone formation, suggesting parallels in mechanisms controlling these processes. Stoeger's molecular data support the previous studies done on rats showing that BMP-2 induced ectopic bone formation in mice undergoes bone development involving both modes, endochondral and intramembranous ossification, simultaneously at different sites of the implant.[25]

A study conducted in Japan[17] analyzes the steps involved in ectopic bone formation. The purpose of the study was to investigate the characteristics of ectopic chondroid/bone matrix and chondrogenic/osteogenic cells induced by recombinant human BMP-2. Five micrograms of rhBMP-2 combined with atelocollagen (used as the collagen matrix) was implanted into the calf muscle of rats and removed on days 7, 10, 14, 21, or 28. Tissue sections were examined using: (i) hematoxylin/Alcian blue/Sirius red stain, (ii) enzyme histochemistry for alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase activity, (iii) immunohistochemistry for types I, II, and X collagen, and (iv) electron microscopy. On day 7, numerous fibroblast-like cells with ALP activity were present on the pellet rim. By day 10, chondroid matrix (CM) had formed, containing both type I collagen and proteoglycans, and often continued into the BMP pellet. On day 14, bone-like matrix formed around hypertrophic chondrocytes as part of

the endochondral ossification process. Co-expression of types I and II collagen within chondrocytes and osteocytes was observed throughout the course of the experiment. These results suggest that fibroblast-like cells invading the pellet differentiate into chondrocytes and form CM under the scaffold of the carrier component. It appears that some chondrocytes change their phenotype to produce the bone-like matrix and remain within the endochondral bone. This process enables rapid osteogeneis to occur.[17]

Another study done in Japan[22] attempted to chemically engineer a substance in order to prolong the calcification of ectopic bone. A synthetic peptide corresponding to residues 73-92 of BMP-2 was investigated. This peptide bound to a BMP-2-specific receptor elevated both alkaline phosphatase activity and osteocalcin mRNA in a murine cell line. The 73-92 peptide also induced ectopic calcification when conjugated to a covalently cross-linked alginate gel. The 73-92 peptide-conjugated alginate gel showed prolonged ectopic calcification for up to 7 weeks in rat calf muscle. In contrast, rhBMP-2-impregnated collagen gel showed maximum ectopic calcification at three weeks, and the calcified products that had formed disappeared after 5 weeks. Histological examination showed that the 73-92 peptide-conjugated alginate gel induced many osteoblast-like cells and few osteoclasts. In contrast, rhBMP-2-impregnated collagen gel induced many osteoclasts. These results suggest that the 73-92 peptide on alginate gel remains active at the implant site, continuously inducing differentiation of osteoblast precursor cells into osteoblasts, and activates osteoblasts to promote ectopic calcification.[22]

Another study used implants of collagen combined with adenoviral vector containing the BMP-2 gene (AdBMP-2) placed subcutaneously in rats to induce bone

formation. In this same study they also evaluated whether targeting the AdBMP-2 vector through an alternative pathway, fibroblast growth factor (FGF), would increase the vector's potency. It was found that local delivery of AdBMP-2 in a collagen matrix rapidly induced bone formation within 14 days, and targeting the virus through FGF receptors significantly enhanced the osteogenic potential of AdBMP-2.[23] This research suggests that combining the effects of growth factors might induce a synergistic effect on bone formation.

BMPs have also been used in distraction osteogenesis. One experimental study in sheep performed alveolar ridge augmentation by distraction osteogenesis using BMP-2 to enhance bone regeneration, followed by implant placement. The average amount of distraction was 12 mm, the distance was held for 12 weeks, then a biopsy was taken. Then titanium cylindrical implants were placed in the newly augmented bone. The histological study demonstrated that the association of DO and BMP-2 resulted in increased trabecular bone size and volume. They found that alveolar distraction augments atrophic ridge and creates new bone that permits implant placement. rhBMP-2 enhances bone quality and may shorten the consolidation period of distraction allowing for earlier implant placement.[20]

Distraction osteogenesis was used in combination with BMP-2 on the posterior alveolar ridges of *Macaca fascicularis* monkeys. One side of the mandibular served as a base line without the use of BMP, and the other side was injected with a BMP cytokine into the distracted area using a collagen carrier.[3] After eight weeks the two sides were compared and the side where BMP-2 had been used showed accelerated bone healing. This study showed that the combination of distraction osteogenesis and BMP-2 produce

maximum bone enhancement of the distracted segments, which in this case produced an increased height of the alveolar ridge without the use of bone grafting.[4]

Two-month-old minipigs were used in another study to observe the rate of growth of the cranium after a one-sided defect of the parietal bone (including the coronal suture) was generated. One group was not treated, the second group had an autogenous iliac bone graft, and the third group was repaired with rhBMP-7 collagen (500 mg/g) and carboxymethylcellulose. After four months, the first group had major bony gaps remaining, the second group had minor bony gaps remaining with significantly less bone volume than the non-defect side, and the third group had bony continuity in all cases with no significant differences in the treated vs. non-treated sides. Skull diameters increased by 16.4%, and the physiological centrifugal cranial expansion remained undisturbed.[26]

A research team in Australia set out to evaluate the role of BMP-2, BMP-4, and TGF-beta superfamily related postreceptor signaling glycoproteins Smads 1-5 in distraction osteogenesis. (Smads are the intracellular modulators for the extracellular binding of TGF-beta superfamily molecules to the serine/ threonine kinase receptors.) They used 12 sheep; 6 had their mandibles distracted 1mm/day, and 6 were distracted 4mm/day. Each group was distracted 24mm, and then allowed a five-week fixation period. No significant difference between the two groups was noted. In fracture healing, BMP-2 and BMP-4 have been localized in the cambial layer of the periosteum, where healing occurs by intramembranous ossification. This study supports induction of BMP-2 and BMP-4, their related smads 1-5, and intramembranous bone formation associated with mechanical strain in distraction osteogenesis.[10]

Maxillary bone deficiencies, such as cleft palate and underdeveloped maxilla that require bone graft or regeneration after orthopedic or surgical expansion, pose a significant biochemical burden. To accelerate bone formation in acutely distracted palatal sutures, rat organ cultures were stimulated with Nell-1 or BMP-7 for 8 days in vitro. Cowan *et al.* hypothesized that Nell-1 stimulation of the distracted palatal suture would accelerate bone formation. Alcian blue staining was used for cartilage localization. While both BMP-7 and Nell-1 produced significantly more bone and cartilage, BMP-7 induced both chondrocyte proliferation and differentiation, while Nell-1 accelerated chondrocyte hypertrophy and endochondral bone formation. This suggests distinctly different mechanisms that produce similar results.[8]

In a study by Chen[6] the expression of BMPs was monitored in 12 cats at 2, 4, 6, 8, or 12 weeks. Cleft palate defects were distracted at a rate of 0.4mm 2/day. BMPs were expressed as early as 2 weeks, with the most extensive expression in the 4-6 week range, gradually tapering off in the 8-12 week range.[6] This study gives a good base line for what occurs naturally with regards to BMP expression.

Another variable to consider is the age of the subject when using BMPs. While it has been shown that 20 year-old monkeys (equivalent to the 80 year-old clinical patient) can regenerate bone comparable to that of an 8 year old or middle aged monkey, it has been reported in the literature by Fleet *et al.* in 1996 that animal aging decreased the amount of rhBMP-2 induced bone in rats. It was postulated that this decreased induction may be due to a decrease in the number of mesenchymal stem cells in older rats, or to a change in the response of the target cells to BMP-2.[5]

A review of literature on BMPs revealed a number of interesting findings. For example, mice deficient in BMP-2 have developmental abnormalities of the skull, hind-limb, and kidney. A dose-dependent occurrence of cyst-like bone voids was also noted in these animals. Other studies showed no relation between the concentration of BMP-2 and the amount of bone formed.[14] The biomechanical performance of the defects that had been treated with the lowest dose of rhBMP-2 (150 mg) was superior to that of the defects that had been treated with the higher doses, and this finding was attributed to the lack of cyst-like voids in the lower dosed animals. This evidence suggests that the dose of rhBMP-2 protein may have to be adjusted for different clinical applications.[14] In another study[14], the presence of voids in the fusion mass was noted in two cases in which either an open-cell polylactic-acid polymer or a polylactic-polyglycolic acid carrier was used. The authors concluded that further study should be conducted regarding the influence of the protein dose and a specific carrier on the formation of these voids. Although these preclinical studies have been promising, the relatively high doses of rhBMP required to induce adequate bone formation suggests that large amounts of recombinant protein may be required to produce a clinically important effect. The carriers that have been tested most frequently are a collagen matrix, demineralized bone matrix, and synthetic polymers. It is unclear whether these are the best vehicles for presenting BMPs (or growth factors) to receptors or responding cells.[14] Lieberman goes on to say that he believes that fibroblast growth factor is the most clinically promising stimulator of bone repair (as opposed to BMP), saying that a dose response is not found suggesting a threshold amount of rhFGF-2 will enhance bone-repair, but a higher dose

will not improve healing.[14] It is worth noting that BMP-2 and BMP-7 have received FDA approval for human clinical uses and have entered the market on a global level.

In the process of healing a bone fracture a callus is formed. Distraction osteogenesis takes advantage of the fiberous callus and stretches it about 1mm/day to gain length. The rate of bone callus formation with DO is said to be about twice that of fracture gap healing.[19] Without the use of BMP, it is standard practice to wait one sometimes two weeks for the callus to form after an osteotomy is done.[3] A study done in Japan[19] evaluated the callus formation in the femur of rabbits with distraction osteogenesis. A significant increase in callus formation was found in the group where BMP and a polymer-coated gelatin sponge (carrier) were used. These rabbits were subjected to DO immediately after surgery, and still they exhibited good callus formation 2 weeks some in only one week after surgery. Afer six weeks, the formation of bone cortex and marrow cavity was almost complete, where as in the other specimens where no BMP was used only a good callus had formed and the cortex was thin. Torsional strength tests confirmed the maturity of the bone where BMP was used.[19] It has been suggested that only 3-4 days are necessary for recovery of the periosteum and less than one week for that of the intermedullary blood supply to take advantage of the higher rate of BMP to accelerate callus formation.[21] The concentration of rhBMP-2 with PGS has been shown to be gradually reduced into half after 3-4 days, 10% after 10 days, and 0.5% three weeks after osteotomy. Thus, rhBMP-2 with PGS exhibited sustained release for up to three weeks.[24] Without any delivery system, rhBMP-2 injected locally remained in place for about 7 days.[1]

Histologically, DO exhibits mainly intramembranous ossification, which occurs in the central portion called the fiberous interzone.[18,31] By contrast, endochondral ossification occurs at either end of the distraction site after the invasion of the vessels from the intramedullary tissue. Yasui et al. found that intramembranous ossification occurred mainly during DO, but endochondral ossification occurred only during the early phase.[18,31] It was suggested that this may be due to the higher concentration of rhBMP-2 in the early phase.[19]

CHAPTER THREE

MATERIALS AND METHODS

Surgical procedures were carried out in a special room in an animal care facility at Loma Linda University, staffed by a fulltime microsurgery technician, John Chrisler who performed all surgical procedures on the rats. Micro CTs and the micro MRI were performed by Dr. Andreas Obenuas and Anna Smith. The titanium cages were constructed in two different labs; the non-expansion cage was made from titanium mesh (Osteomed) that was welded to form custom cages by Dr. William Nordquist, DDS, and the distraction cages were cast titanium wire cages that were engineered specifically to attach to the distractor (KLS Martin) with screws (American Orthodontics). These cages were designed by Andrew Christiansen and Nick Flannery at Medical Modeling. Medtronic supplied and Jonathan Brunger prepared the concentration of rhBMP-2 prior to each set of surgeries, histological slides were done by John Hough, while the TRAP stain (Sigma) was prepared by Dr. Ken Wright. A list of supplies and instruments can be found in Figure 1. Figure 2 shows a sterile set of instruments with two non-expansion cages in sterile saline ready for surgery. Figure 3 shows the distraction cage apparatus fully assembled.

12mm titanium distractor fabricated by KLS Martin
Two cage types:
1) Non-distraction cages fabricated by William Nordquist, DDS
24mm x 8mm x 6mm of titanium sodered mesh from OsteoMed
2) Custom designed to fit the distractors, cast titanium distraction cages each
10mm x 8mm x 6mm fabricated by Medical Modeling
Four screws or locking pins to attach the distractor to the two cages fabricated and customized by American Orthodontics
ACS (Absorbable Collagen Sponge) One sponge for the non expansion cages, 1.5 sponges per expansion cages from Medtronics
1.4mL of 0.3mg/mL rhBMP-2 per sponge from Medtronics
Rats white SD 290-415g
4-0 Vicryl P3 or Pe3 suture
4-0 Proline Ps3 suture
#15 Bard Parker blade
Scissors
Hemostats
Variety of forceps
Tuberculine syringes
25-27gage x 1in needles
Triple antibiotic ointment
Opthalmic ointment

Figure 1 List of essential instruments for initial surgery.

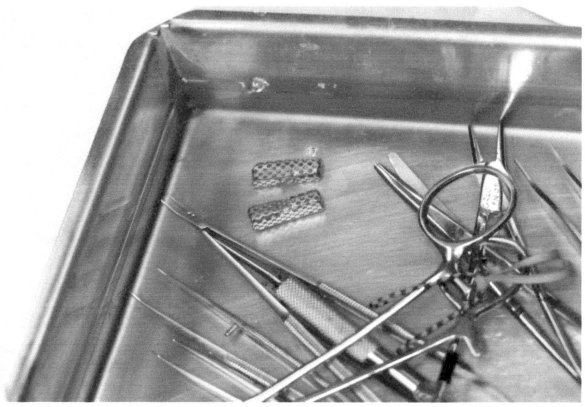

Figure 2 Instruments and cages in sterile saline.

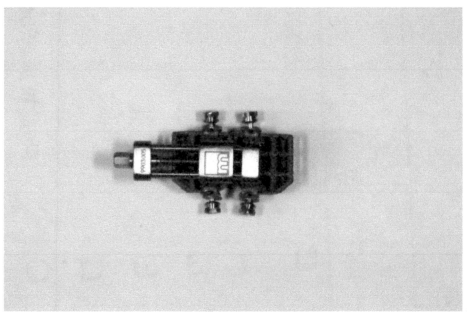

Figure 3 Distraction cage.

Rat Selection

Step 1

Rats were selected by size and sex in order to have a standardized group without extremes. Male Sprague-Dawley rats weighing 290-415g grams were used in this study, with the average weight being approximately 325g.

Implant cage preparation

Step 2

In this study, both the screws and the cages to be implanted in the rats were custom made and needed some modifications prior to assembly. The threaded portion of the 2mm diameter screw was ground flat on two sides. Then just below the screw head the threads remaining were shaved down with a bur to make a square midsection 1mm x 1mm for about 1.5-2mm in length along the shaft. By leaving the end rectangular, the screw acted like a locking pin. One quarter turn of the screw kept the arms of the distractor snug against the drilled out slot in the cage that was just large enough for the rectangular end of the screw to pass through. All the pieces were assembled prior to sterilization of the parts as shown in Figure 4, to make sure no further modifications needed to occur. The distractor attached to the cage was tested for its ability to expand. This was because the cage would get hung up on the distractor at around 4mm of distraction and no further expansion could occur. The necessary modifications were made and the cage was sterilized along with the distractor, screws already assembled and Howe pliers and screw driver to put it back together if it came apart post sterilization.

The non-expansion cage required no prep or assembly. Figure 5 shows the window where the collagen with rhBMP-2 was placed into the cage.

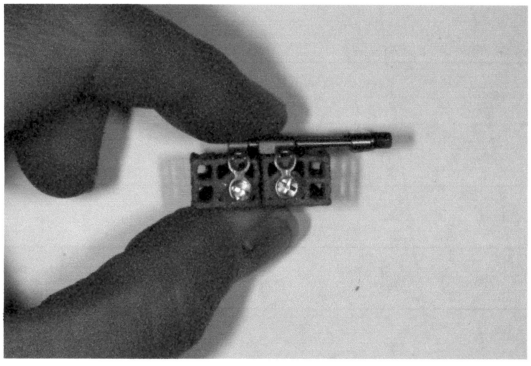

Figure 4 Profile view of the distraction cage.

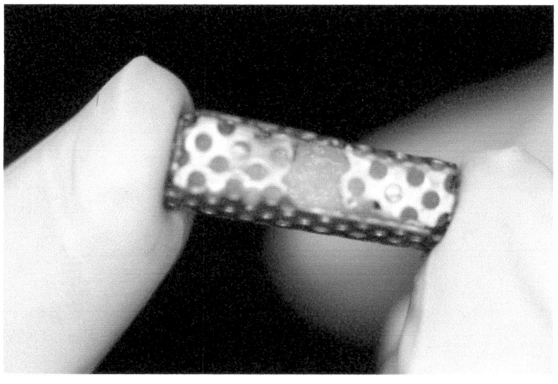

Figure 5 Non-expansion cage containing collagen.

RhBMP-2 Preparation

Step 3

Collagen and rhBMP-2 preparation was the most technique sensitive part of this process. We followed the instructions given by Medtronic, Inc. when possible. For a collagen sponge 1 in. x 2 in. x 1/4in, we added 1.4ml of the BMP solution to the shiny side of the sponge and allowed it to soak on a surface that does not absorb moisture (as shown in Figure 6) for a minimum of 15 minutes and a maximum time of 120 minutes. In order to place the BMP and collagen sponge into the cage without mutilating the absorbable collagen sponge (ACS) it was necessary to cut the collagen sponge prior to soaking it in BMP. The size was determined by cutting the collagen slightly larger than the cage or cells in the distraction cage it needed to fill. After the collagen sponge was soaked in the BMP solution it quickly became like wet tissue paper and fit easily into the cell or cells it was cut to fit in (see Figure 5). The spaces inside the cages were filled with the sponge, but the cages were not overpacked because overpacking caused the BMP solution to be squeezed out.

The dimensions of the distraction cages were 8mm x 10mm x 6mm for each of the two pieces for a totally assembled size of 8mm x 21mm x 6mm. The materials required for a cage this size are 1 ACS (or 3 in x 1 in x 1/4in of collagen sponge) and 2.0ml of 0.3mg/ml rhBMP-2. Only one ACS and 1.4cc of 0.3mg/ml rhBMP-2 was required for the non-expansion cage.

Figure 6 Adding rh-BMP-2 to the ACS.

Rat anesthesia

Step 4

Rat anesthesia was done two ways. Phenobarbital was not used because of the risks of overdose and the long recovery time. Once a technique for placement was established, it became much more practical to use 5% Isoflurane initially, and when the rat was asleep the Isoflurane was reduced to 2-2.5% depending on the tolerance of the rat. The cone used for the delivery of the Isoflurane is shown covering the rat's face in Figure 7. This way, the initial anesthesia was rapid, profound, and had a large margin of safety with an almost immediate recovery. The only drawback was the increased blood flow at the surgical site. Initially, Ketamine 80mg/ kg and Xylazine 6mg/ kg were given IP as shown in Figure 8. A maintenance dose of 20mg or 0.1cc of Ketamine was used if the rat began to wake before the procedure was done or if the rat had built up a tolerance from prior administration of these drugs from the prior surgery. Ophthalmic jelly was

placed on the rats eyes, particularly when using Ketamine because this drug dried out the rat's eyes rapidly.

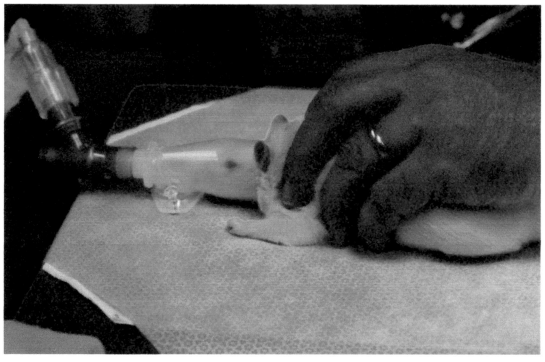

Figure 7 Inhalation anesthesia

Figure 8 Anesthesia given IP

Surgery Preparation

Step 5

The site was shaved prior to the incision, and a hair removal cream was used for a short time so the skin did not become too irritated. The site was sprayed or doused with Betadine® solution. All instruments used were sterilized by autoclave, and a sterile field was prepared to perform the surgery. We wrapped tape around the feet of the rat, and tacked the tape to a surgical board away from the body of the rat as shown in Figure 9. The cone used to deliver the 5% Isoflurane to the rat was adjusted and tacked down to optimize anesthesia delivery to the rat.

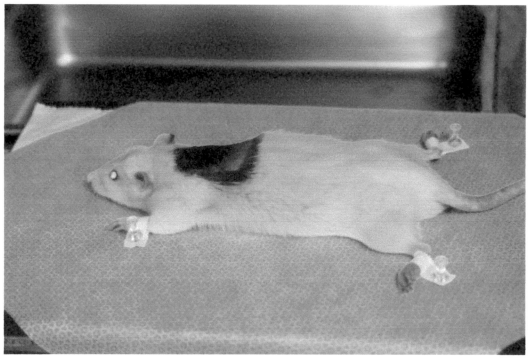

Figure 9 Rat prepared for surgery.

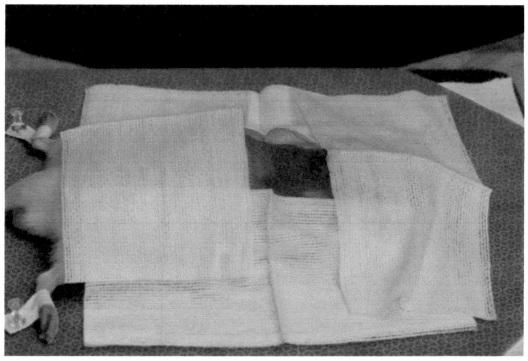

Figure 10 Coronal incision.

Cage Placement

Step 6

A coronal incision was made just behind the scapulae for the non-expansion cages shown in Figure 10 and 11, while a lengthwise incision of about 3cm was made for the expansion cages as shown in Figure 12. The incisions were made through both the thick skin and the investing fascial layer giving access to the spinotrapezius muscle. The two anterior corners of the cages were tacked with one nylon suture to each side of the exposed muscle. A double layer closure was done to encapsulate the cage. The first closure was the muscle's fascial layer under which the cage is implanted. The second closure was the skin layer.

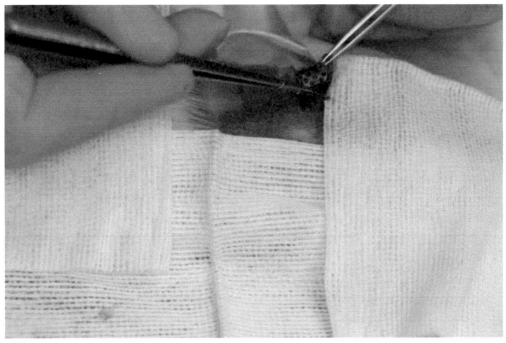

Figure 11 Mesh cage placement.

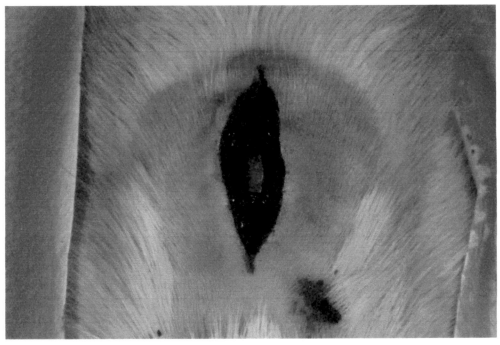

Figure 12 Distraction cage placed.

Around day 7 or 8 after implantation a seroma, like the one shown in Figure 13, had formed around the implanted cages with the BMP-2 in all but one rat. If the seroma appeared to cause discomfort, it was reduced by aspiration of the fluid surrounding the cage. Between 5-7cc of fluid was aspirated in one group of (three) rats.

Figure 13 Seroma formation.

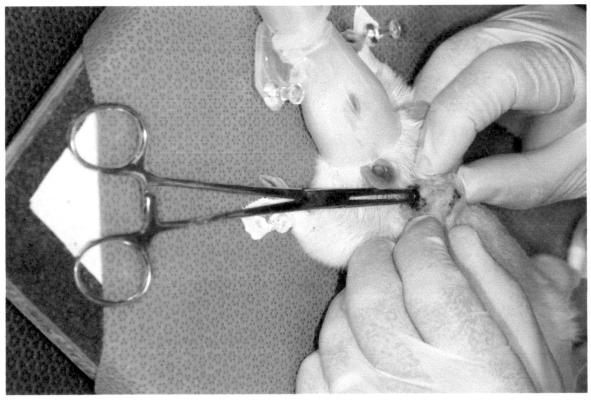

Figure 14 Hemostats attached to knob.

Distraction Osteogenisis

Step 7

By this time(specify time) a thick capsule formed around the implanted cage and in all cases some serum was present within it. An incision was made the length of the cage to access the tissue between the two cages. The tissue was then cut with a #15 Bard Parker blade and the wound was closed. Twenty-four hours later, a small incision was made just wide enough to access the knob of the distractor with a hemostat. The hemostat locked on to the distractor knob is shown in Figure 14. Beginning on day 15 post-implantation, the distractor knob was turned 2 full turns clockwise once a day for 7 days to expand the cage 2/3 mm per day. Expansion was halted after 7 days (day 22 post implantation) and held for one or two weeks.

Intravital Fluorescent Labeling

Step 8

Intravital fluorescent labeling by tetracycline was used as a bone marker to show whether bone was still forming or mineralizing. Since the peak calcification is reported to be approximately three weeks[16] after implantation, a tetracycline marker was administered at that time. If the bone was still mineralizing, the tetracycline would incorporate into the forming bone matrix, thus causing the bone to fluoresce when viewed under an ultraviolet light. This method was also useful in telling where the bone was forming during the 3-4 days it took to clear the tetracycline after injection. We administered 15-20mg/kg tetracycline in sterile water solution to a total volume of 3-4mL

intraperitonially (IP) at three weeks. In the next study group, oxytetracycline will be administered 15-20mg/kg in 3-4ml of sterile water IP at five weeks to produce a contrasting color of fluorescence and ascertain whether or not the bone is still regenerating.

Micro-CT

Step 9

The CBCT images were taken anytime a change in calcification was expected. An initial CBCT was done on all seven rats to show initial bone density. Change in density was observed in one week intervals after the three weeks it took to grow bone. Since there is possible damage caused to bone formation with every CBCT taken, no more than three CBCTs were taken per rat, and done at least one week apart. By taking serial CBCTs, relative density can be assessed with out surgical intervention. We obtained CBCT data within 24 hours after placement, at (post-expansion) three weeks, and at four weeks after implantation.

Micro-CT Equipment

All exposures were performed using a micro X-ray computer tomography (micro-CT) unit (MicroCAT II®, ImTek Inc., Knoxville, TN) with an 80 kVp X-ray source and a reconstruction volume resolution as low as 15 microns (ImTek, Inc. specifications). The scanner is a "step and shoot" type system, which rotates a low-

power X-ray source and an X-ray detector array about the specimen. Data acquisition is controlled by the MicroCAT II® software, which operates on a Windows XP® work station. X-ray data sets are stored on the system computer hard drive and reconstructed simultaneously with the acquisition of the scan.

Micro-CT Data Acquisition

Each animal was scanned, while anesthetized with 2.5% Isoflurane, using 75 kVp at 1000 μA with 360 projections per scan. The CCD camera exposure time for each projection was 200 msec. The source to center distance was 386.14 mm and the source to detector distance was 511.14 mm. One positioning radiograph and 25 light: 25 dark calibration measurements preceded each scan.

Micro-CT Image Reconstruction

All micro-CT images were acquired using a CCD field width of 2048 pixels and length of 2048 pixels with 4x4 data binning. The images were reconstructed using a Shepp-Logan filter and real-time reconstruction. Reconstructed volumes were 512 x 512 x 512 voxels with 0.126 mm voxel dimensions. All reconstructed images were visualized using Amira® software (TGS, Inc.)

Specimen Retrieval and Processing

Step 10

Surgical cage removal for both cage types was done with a straight incision slightly longer than the cage as shown in Figure 15. The capsule had become thick and

tough to cut through at this point. The cage was dissected from the surrounding soft tissue and removed as shown in Figure 16. After checking for bone with a needle tip the cage was immediately placed in 10% neutral buffered formalin fixative solution for a minimum of 24 hrs. The cage was then cut in half with an Exakt diamond band saw to allow the formalin to fully perfuse the tissue.

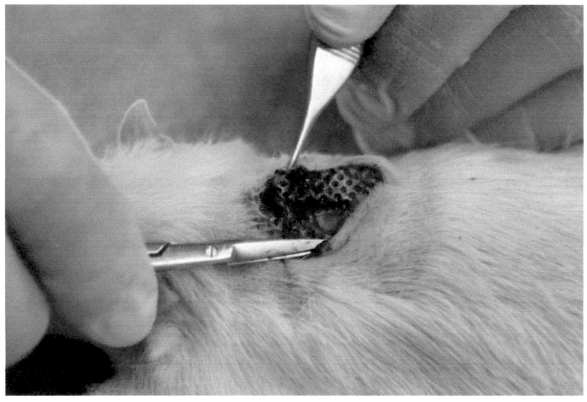

Figure 15 Mesh cage removal.

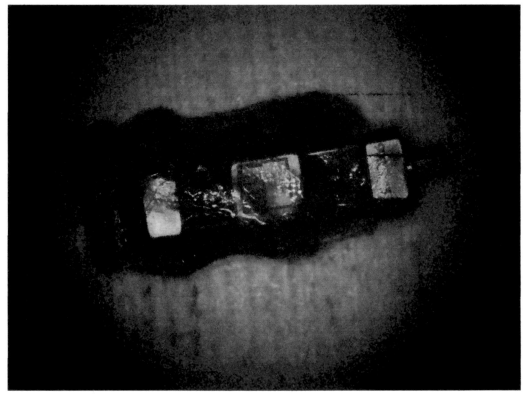

Figure 16 Distraction cage removed.

Analysis of Specimens

Step 11

Analysis of the specimens was done with CT three dimensional reconstruction, and gross visualization. Histological analysis was done on only half of each specimen. The other half was preserved for gross observation and preservation of the tetracycline induced fluorescence.

Histological analysis

Step 12

Decalcification was done with 15% neutral buffered EDTA for histologic analysis. It was necessary to decalcify the bone to be able to peel it out of the titanium cage and cut it 5 microns thick without fraying the tissue. The bone was submerged in 3L of 15% neutral buffered EDTA for two weeks (one week was not long enough), then processed for paraffin embedding. The stains used were the standard H & E (Hemotoxin and Eosin) and a TRAP (Tartrate Resistant Acid Phosphatase)(Sigma) histochemical method to stain osteoclasts.

CHAPTER FOUR

RESULTS

Marked bone formation occurred in all 6 rats. A concentration of 0.3mg/ml rhBMP-2 formed considerably more bone than did a concentration of 0.1mg/ml. The concentration of rhBMP-2 affected the tissue type formed. In the cage with the 0.1mg/ml concentration multiple tissue types were formed; some bone, some tendon-like tissue, some dense irregular connective tissue, some areas were highly cellular, and some areas of bony matrix were devoid of a single cell. In one area, the (presumed) bone appeared in thin spicules, forming a reticular pattern that is unlike any tissue recognized in the human body. The samples were all highly vascular. Bone formed continuously across the 4 mm expanded portion. Viewing it grossly, bone appeared only along the base of the cage when observed 7 days post expansion (or a total of 28 days post implantation). A dense connective tissue capsule formed around the implanted expansion cage. There was evidence of hyaline cartilage formation within the muscle's fascial layer that encapsulated the cages. Surrounding the hyaline cartilage were cells with the same orientation as one would expect to see in tendon or ligament (or a thick periosteum). On the outer edge of the cartilage were small areas that appeared to be transforming into bone. A tissue sample of the capsule was taken at the time of cage removal at 28 days post implantation. It was harvested near the site that was re-opened once a day for 7 days to turn the distraction knob. Within this capsule was some cartilage adjacent to some bone with a (presumed) periostium/perichondrium

surrounding it. On the same slide was found another (presumed) periostium capsule filled with fat cells.

The optimal concentration of the rhBMP-2 in solution for a rat of this size was found to be 0.3mg/ml. When we tried to conserve BMP by soaking another sponge in the liquid remaining from the 1.4cc added to the first sponge, significantly less bone formed. The liquid that was left over likely had a lower concentration of BMP than the initial solution because much of the BMP had bound to the collagen sponge leaving behind the neutral buffer solution

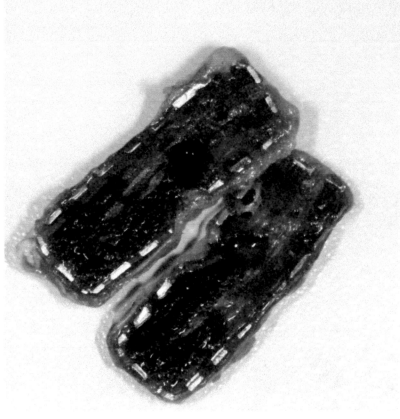

Figure 17 Bone formed with 0.1mg/ml concentration rhBMP-2 with cyst-like voids harvested at 3 weeks.

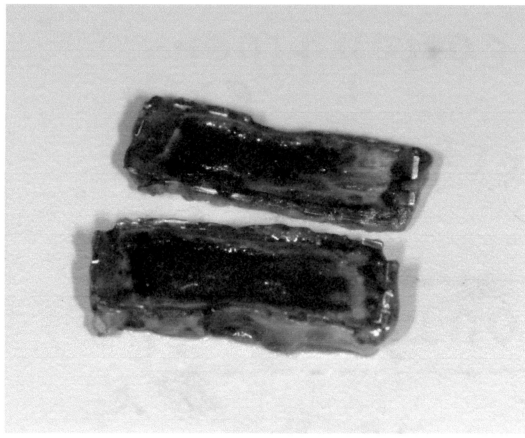

Figure 18 Bone formed with 0.3mg/ml concentration rhBMP-2 harvested at 3 weeks.

Figure 17 shows unfixed sections of the specimens from the pilot study were 0.1gm/ml rhBMP-2 was used. Note the fluid filled vacuole (or void) in the center of the cage. A cage from an animal using 0.3mg/ml rhBMP-2 is shown in Figure 18. Note the dense bone formation along the whole inner border of the cage. Both were removed on day 22 postimplantation.

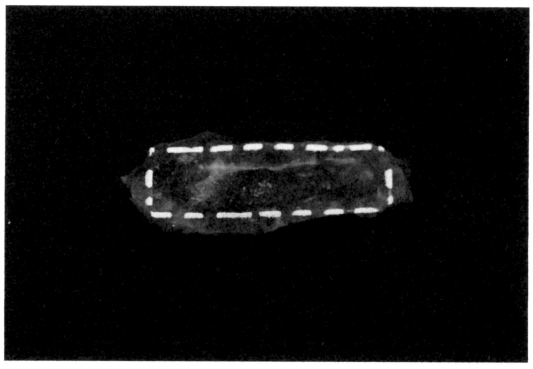

Figure 19 Bone formed with 0.3mg/ml rhBMP-2 harvested 24 hrs prior to this photo on day 25 after implantation.

Figure 20 Intravital fluorescent labeling shown under a UV light. The yellow/green area is the bone that formed after three weeks in a distraction specimen cut in half.

Figure 19 is a raw cut of the bone formed with 0.3mg/mL rhBMP-2, removed at 25 days post-implantation. Notice the cortical bone forming outside the cage and a marrow cavity in the center. Figure 20 is a distraction cage cut in half under a UV light to expose the bone that took up the tetracycline marker given at 3 weeks. The

yellow/green area is bone that mineralized after 3 weeks. The blue is the UV light reflecting off of bone formed prior to 3 weeks. In the center is the immature distracted bone or callus not yet fully mineralized. This cage was removed at 4 weeks post implantation. Note the difference between the composition of the centers of the two cages. There is a marrow cavity in the non-expansion cage, and dense bone in the center of the two halves of the distraction cages.

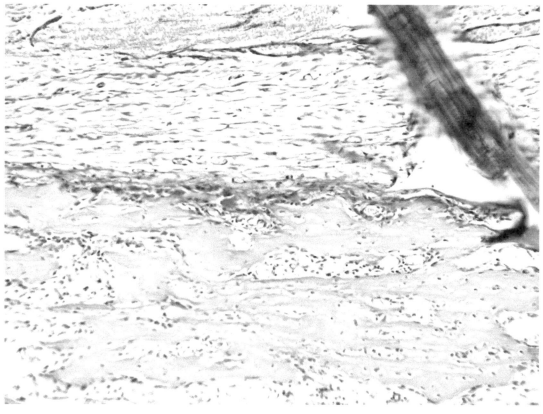

Figure 21 TRAP stain (red cells) in a 0.3mg/ml rhBMP-2 specimen showing osteoclasts between periosteum and intramembranous bone.

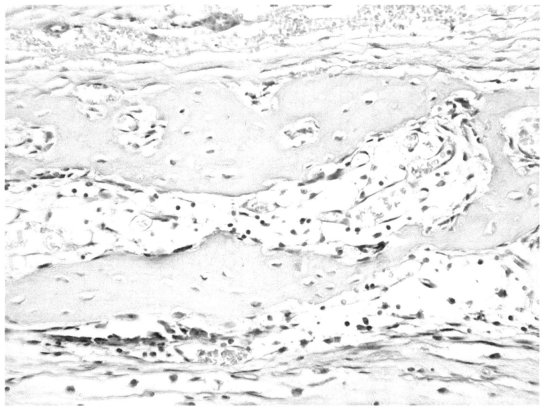

Figure 22 TRAP stain in a 0.1mg/ml rhBMP-2 specimen showing osteoclasts (dark red cells) along endochondral bone.

Above are sections stained for TRAP to show osteoclast activity (osteoclasts are red). Figure 21 is a 0.3mg/mL rhBMP-2 specimen at 20x showing osteoclast activity between the cortical bone and an apparent periosteal layer. Figure 22 is a 0.1mg/mL rhBMP-2 specimen at 40x showing osteoclast activity on a small spicule of bone in the center of the cage. Stages of both intramembranous and endochondral bone formation could be observed histologically. Osteoblasts can be observed along the borders of the bone.

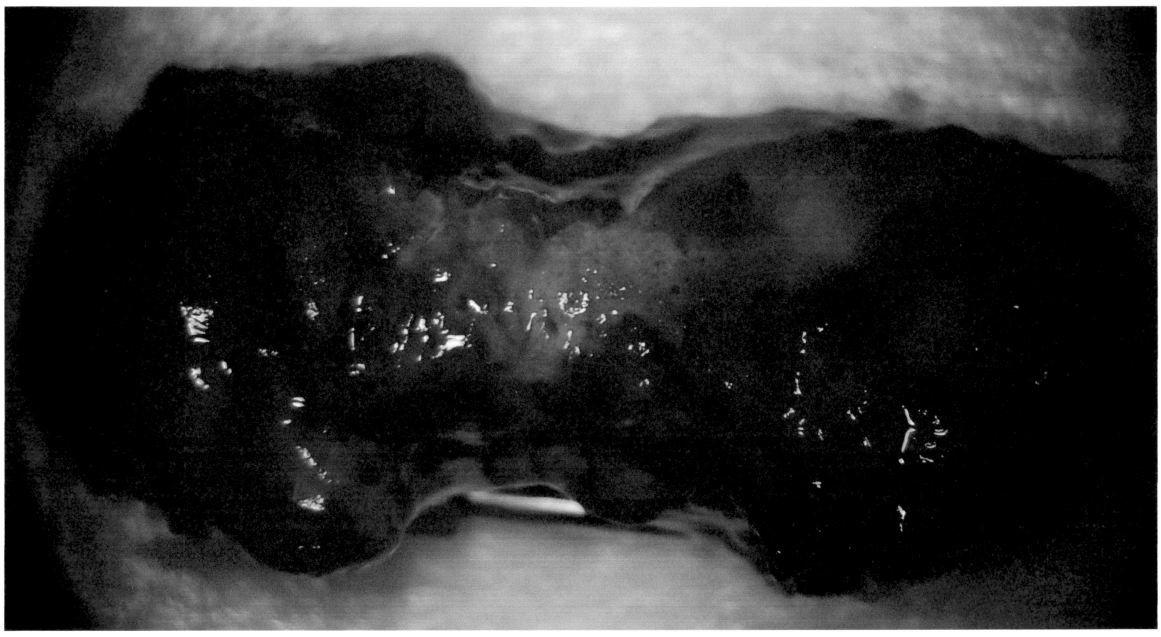

Figure 23 The bottom of a 0.3mg/ml rhBMP-2 distraction specimen harvested 28 days post implantation. Note dense bone formation continuously between the two cages just one week post expansion.

Figure 23 is the distraction cage right after removal at 28 days with the 0.3mg/mL concentration rhBMP-2. The soft tissue was scraped away with a 15 blade to show the continuous bone formation along the base between the two cages.

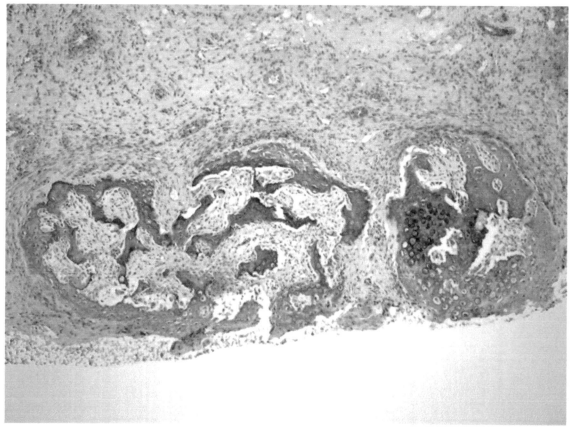

Figure 24 Endochondral bone formation in the muscle fascial layer.

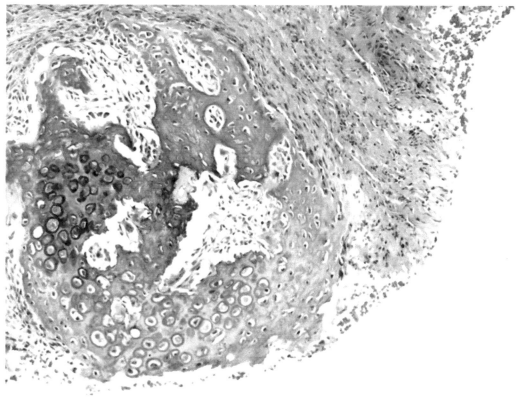

Figure 25 Magnification of 40x showing endochondral bone formation. Note the chondrocytes in the center and bone forming on the periphery.

Above is a sample taken from the capsule which formed around the implanted cage. Figure 24 is shown at 10x, and figure 25 at 40x to show the chondrocytes in the center and the oseocytes at the bony periphery. This dense irregular connective tissue fascia now contains a uniform orientation of cells surrounding the islands of cartilage and

bone. This layer will likely become the periosteum surrounding the forming bone, which is going through a cartilage phase.

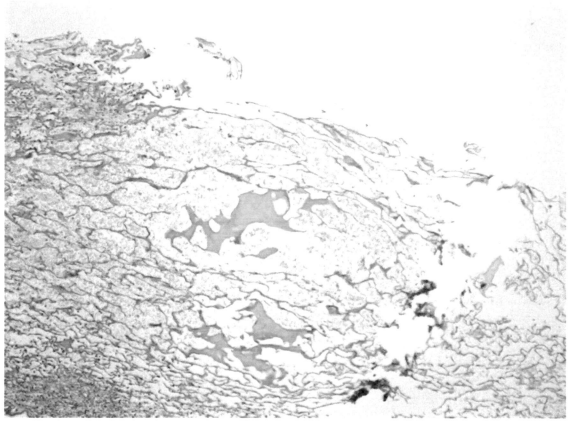

Figure 26 Tissue not found in the human body. This section was taken from the 0.1mg/ml rhBMP-2 specimen. It is possible it could be the collagen matrix and red blood cells

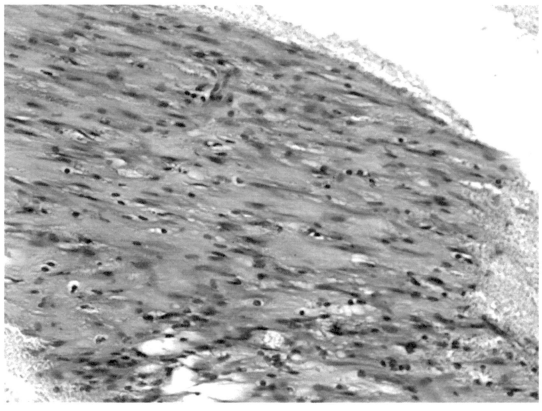

Figure 27 This tendon-like tissue was found adjacent to the cyst-like void in the 0.1mg/ml rhBMP-2 specimen.

Figure 26 and 27 are both sections taken from the specimens formed with 0.1mg/mL rhBMP-2 to show the variety of tissue types formed. Figure 26 shown at 10x is unrecognizable tissue with very thin, reticular matrix devoid of cells. It is surrounded by red blood cells. It is likely that this is the collagen sponge. Figure 27 is what appears to be tendon at 40x. The cells are all oriented in the same direction, and there is no bone in this area of the slide. This tissue is near the fluid filled void seen in Figure 17 with many red blood cells adjacent to it.

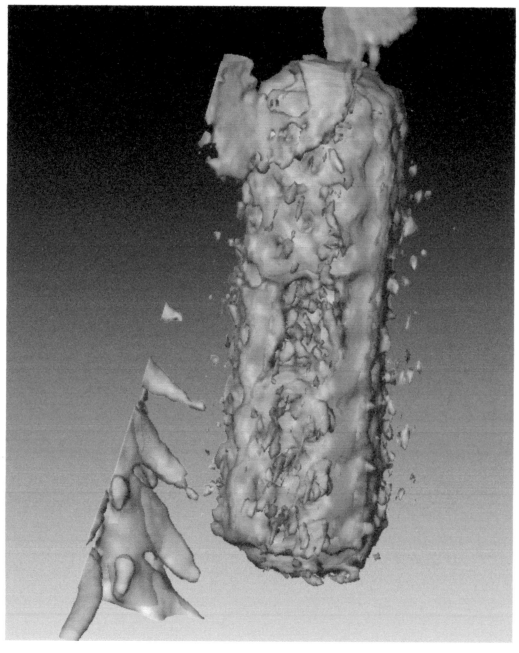

Figure 28 Three-D reconstruction of the CBCT image taken of the non-expansion specimen on day 21 post implantation. Bone of the spine and scapula are included for relative density reference.

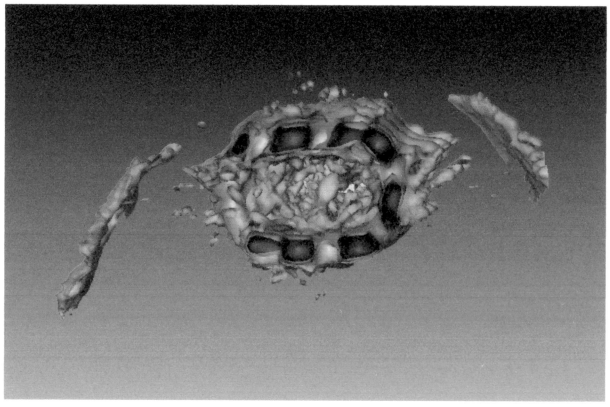

Figure 29 Same as Fig.28 only cut in the center to view inside the cage. Both scapula are shown for relative bone density reference.

Above are 3-D reconstructions using CT images taken on a 0.3mg/mL rhBMP-2 animal with a non-expansion cage just prior to removal at 25 days. In Figure 28 the cage has been made somewhat transparent in order to visualize the bone and some cartilage formation. Note a section of the rat's spine and scapula for comparison. Figure 29 is to differentiate the bone densities formed in and around the cage. Note the bone that is the densest formed adjacent to the titanium mesh as seen by the transparent orange color. The image was set to show a minimum density equivalent to that of the rats own skeleton. The large sections of floating bone are the scapula of the rat.

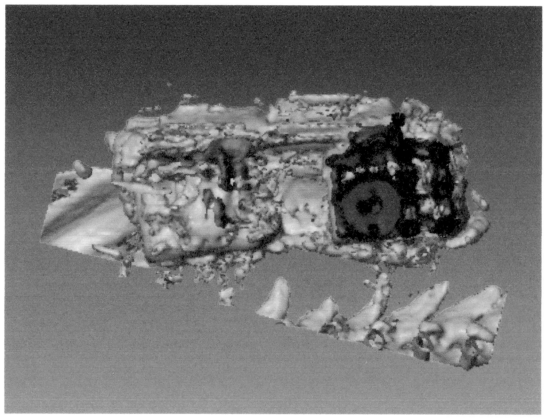

Figure 30 Bone formed in distraction specimen 7 days post expansion. CBCT was taken 28 days post implantation. Relative densities are color coded. The tan color is the normal bone density, the red color is very dense bone that likely overlaps with the titanium cage, and the blue is strictly the titanium.

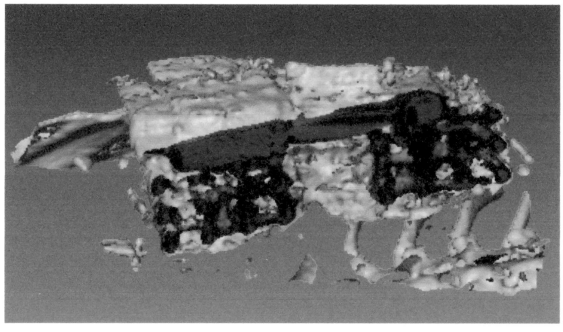

Figure 31 Same specimen as in Figure 30, only cut through the center and rotated to view about 20 degrees above the horizon. The rat's spine is shown on the bottom and its scapula is shown on the left for density references.

Figure 30 is a 3-D reconstruction of the distraction cage as viewed from the side, while Figure 31 shows the same cage cut in half viewed about 20 degrees above the horizon. The relative densities are 1600-2999 for the bone, 3000-3999 for the very dense bone in red, and 4000 and above for the titanium cage in blue. These parameters were set visually relative to the spine of the rat shown below the cages.

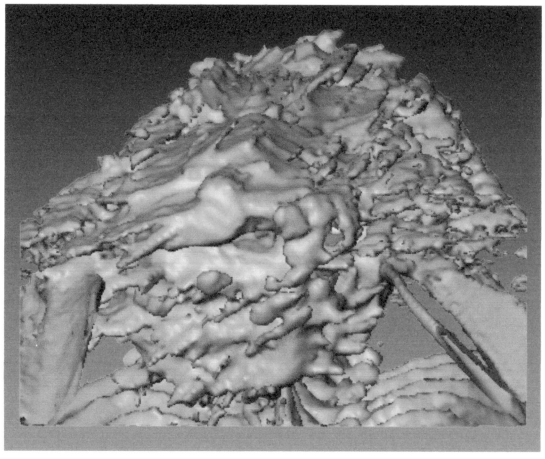

Figure 32 Same as Figure 30 and 31 viewed from above the head of the rat towards the tail to get an idea of the general pattern of bone formation.

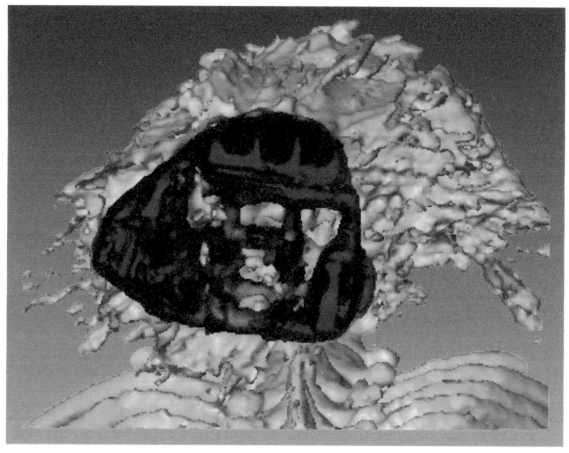

Figure 33 Coronal cut through the first titanium cage.

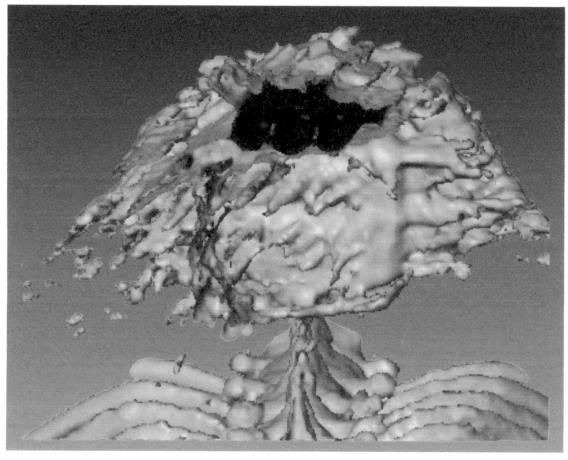

Figure 34 Coronal cut just behind the first cage to show all the bone formed between the two cages being distracted. Note the titanium distractor on the top and the rat's spine and rib cage shown below.

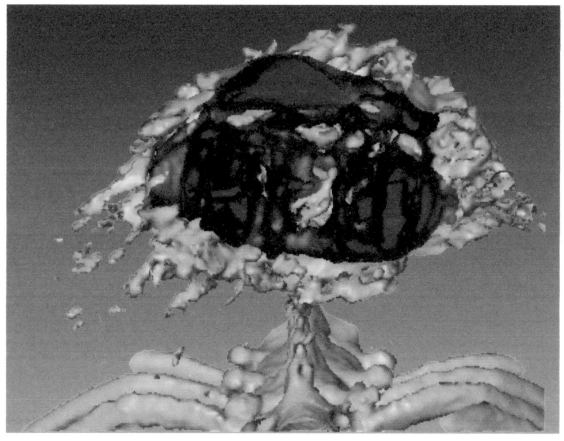

Figure 35 Coronal cut through the second cage at the level of the locking pin to show dense bone formation surrounding the titanium screw.

Figure 32 to Figure 35 are cuts through the same distraction specimen as in Figure 30 and 31 except they are viewed longitudinally. The front of the cage as well as the distractor are covered with a thin layer of bone as seen in Figure 32. The titanium grid of the first half of the distraction cage can be seen in Figure 33. The central part between the two cages where distraction osteogenesis occurred is shown in Figure 34. In the second half of the cage there is a lot of dense bone. The bone surrounding one of the titanium screws can be viewed in Figure 35.

CHAPTER FIVE

DISCUSSION

Perhaps the most clinically significant finding from this study is that it demonstrates the possibility that patients can be their own autogenous bone bank. Furthermore, this study also shows the feasibility of enhancing ectopic bone formation with distraction osteogenesis. This is significant in that a maximum amount of bone can be placed in a given area that the soft tissue can accommodate. The bone can then be expanded through distraction osteogenesis at a rate at which the connective tissue and fascia can regenerate without dehiscence. These findings have many possibilities for use in surgery such as cleft palate repair, in repairing sites of bone loss caused by infection, cancer, and trauma, reconstructive and plastic surgery, orthopedic surgery in general, or simply to enhance cortical bone adjacent to temporary orthodontic anchorage devices. It would be interesting to see if by using rhBMP-2 we could solve the complications caused by chronic use of bisphosphonates. Since it is a problem of signal differentiation between osteoclasts and osteoblasts, maybe by adding rhBMP-2 it will help regain its ability to "differentiate" in intercellular communication. (A possible application in veterinary medicine may be in the treatment and prevention of laminitis in cows and horses.)

The dose concentration of 0.3mg/ml is considered a "mega level" dose for this size of an animal. However, due to the rapid metabolic rate in the rat, it was necessary to use this dose to gain results. The amount of collagen and rhBMP-2 implanted compared to the rats' body mass would also be considered a mega dose. However, the rats were

able to fill this relatively large cage with bone. All rats survived these procedures, except for one (due to a tetracycline overdose caused by a non-calibrated scale).

One interesting observation was an apparent dose-related variation in tissue types. It is well documented that BMP-2 is indicated in the formation of tendon, ligament, bone, and cartilage.[14] We have observed through histology the presence of all of these tissue types in every sample at every observed time.

The fact that the bone formed first, and possibly, only along the base of the distracted cages would not surprise those who have studied contour formation of bone, or those describing the piezoelectric effect of matrix surfaces. According to one study done in vivo, the changes in electrical fields at the bone surface are predominantly those originating in the muscle, indicating that the local electrical phenomena generated by bone strain cannot be the only factors initiating the cellular response that is responsible for bone remodeling. In fact, the local factors generated by bone strain appear to be over-ridden by the electrical fields generated by the muscle.[16] This may be a factor when considering the fact that the most bone was formed and regenerated by distraction osteogenesis along the surface that was in contact with a major muscle group. An interesting test would be to flip the cage on its back to see if the bone would still form along the base when the distractor is between it and the muscle. Would the bone form and remodel significantly differently?

A possible explanation for the dense cortical bone formation adjacent to the titanium cage could be the influence of geometry on the control of cell growth. Studies have shown that the major difference between inhibited cells and their proliferating counterparts is their shape. Cells in suspension are rounded, whereas cells attached to

plastic (or glass, or steel, and possibly titanium) are flat and spread out. Inhibition of growth in the spheroidal state is characteristic of the majority of normal (untransformed) fibroblasts and epithelial cells. Cells of hematopoietic origin are exceptions and can proliferate while spheroidal and in suspension culture. The "flat" configuration is the signal that gives "permission to proliferate". Isolated fibroblasts in agar, if they are allowed to stretch in one dimension, will grow on glass fibers as thin as 0.05 um. This approaches the dimensions of collagen fibrils.[11]

There were many variables in this study, such as the affect of growing bone in a titanium cage and how that affects the longevity of the bone that is formed, the effect of taking multiple CTs of this forming bone, the rate at which the distraction cage should be expanded, the optimal concentration of rhBMP-2, and the cage design itself. In this study, only the concentration of rhBMP-2 was altered.

Other studies that may follow this one could investigate under what doses and under what circumstances these various tissue types form. More questions arise than are answered by this type of research; for example, is it possible to create a titanium cage that will allow distraction osteogenesis of the spine for crushed vertebrae, spinal infections, or oncologic surgical defects? Is there a localized method to reverse osteoporosis? Could collagen strips laden with rhBMP-2 be used for cosmetic maxillofacial surgery and plastic surgery, (e.g. the bridge of the nose)?

Further studies should be conducted to test the length of time that the ectopic bone remains without resorption. Further quantitative analysis should also be performed on these studies. More samples of the capsule should be taken from various locations to check for consistency of the change in tissue type, specifically cartilage development.

Still other studies should be done to test if the ectopically formed bone can be transplanted to critical areas for repair and survive. Some of these questions will be described in Part II of this study.

CHAPTER SIX

CONCLUSIONS

1. A concentration of 0.3mg/ml rhBMP-2 formed optimal bone in the described rat model at a dose of 1.4cc-2.1ml. A concentration of 0.1mg/ml rhBMP-2 formed multiple tissue types with some, but very little bone at a dose of 1.4ml in a rat model.
2. In this study, bone continued to form between 21 and 28 days in both the distraction osteogenesis and the non-expansion cages. Therefore, it is believed that the cages should be left in the rats for a longer time (4-5 weeks) to observe any difference between non-expansion and expansion models regarding sustained bone formation.
3. The densest bone was observed adjacent to the titanium surface of the cage, screws, and distractor.
4. In the distraction model, bone formed primarily along the base of the cage in the distracted portion.
5. Dense bone formed around the titanium screws by day 28 without any significant forces placed on them.
6. More bone formed outside the titanium cage on the anterior end than the posterior end of the cage in all specimens.

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