Response of induced bone defects in horses to collagen matrix containing the human parathyroid hormone gene

Kristin C. Backstrom, MS; Alicia L. Bertone, DVM, PhD; Erik R. Wisner, DVM; Stephen E. Weisbrode, VMD, PhD

**Objective**—To determine whether human parathyroid hormone (hPTH) gene in collagen matrix could safely promote bone formation in diaphyseal or subchondral bones of horses.

**Animals**—8 clinically normal adult horses.

**Procedure**—Amount, rate, and quality of bone healing for 13 weeks were determined by use of radiography, quantitative computed tomography, and histomorphometric analysis. Diaphyseal cortex and subchondral bone defects of metacarpi were filled with hPTH1-34 gene-activated matrix (GAM) or remained untreated. Joints were assessed on the basis of circumference, synovial fluid analysis, pain on flexion, lameness, and gross and histologic examination.

**Results**—Bone volume index was greater for cortical defects treated with hPTH1-34 GAM, compared with untreated defects. Bone production in cortical defects treated with hPTH1-34 GAM positively correlated with native bone formation in untreated defects. In contrast, less bone was detected in hPTH1-34 GAM-treated subchondral bone defects, compared with untreated defects, and histology confirmed poorer healing and residual collagen sponge.

**Conclusions and Clinical Relevance**—Use of hPTH1-34 GAM induced greater total bone, specifically periosteal bone, after 13 weeks of healing in cortical defects of horses. The hPTH1-34 GAM impeded healing of subchondral bone but was biocompatible with joint tissues. Promotion of periosteal bone formation may be beneficial for healing of cortical fractures in horses, but the delay in onset of bone formation may negate benefits. The hPTH1-34 GAM used in this study should not be placed in articular subchondral bone defects, but contact with articular surfaces is unlikely to cause short-term adverse effects. (Am J Vet Res 2004;65:1223–1232)

Fractures account for 16% of all musculoskeletal injuries in people in the United States annually. Approximately 5% to 10% of the 5.6 million fractures that occur annually have delayed or impaired healing.12 Treatments that could accelerate fracture healing and assure successful union would be clinically beneficial.

Parathyroid hormone (PTH) induces bone formation in humans and several other animals. The anabolic effects of intermittent administration of PTH on bone formation and quality of bone formed have been documented.34,6-10 The amino terminus of human PTH (hPTH) has been isolated; the osteoinductive effects of PTH are localized to amino acid sequence 1 to 34 or 1 to 38,4,17,18 and many other analogues of PTH.3,6,19-24 In particular, hPTH1-34 has positive effects on bone formation in cortical and cancellous bone,23-26 fracture repair,27,28 and osteopenic conditions such as osteoporosis.29-42

Various techniques for administration of gene therapies, including viral and nonviral vectors, have been used to augment healing of several conditions. One such technique that uses a nonviral vector is gene-activated matrix (GAM). Gene-activated matrix is a tissue-engineered construct that has been designed for use in acute injuries involving bones,50-52 skin,53-55 tendons and ligaments,56 cardiac and skeletal muscles,57,58 and cranial nerves.59 The major goal of GAM is to accomplish gene transfer for the expression of approximately physiologic amounts of a recombinant protein for prolonged periods by local cells involved in wound-healing cells.60 Plasmids are used to transfer genes for growth factors and cytokines, rather than to deliver recombinant proteins.61 Plasmids were chosen for gene transfer because plasmid diffusion was not expected to cause systemic toxicosis because of the high efficiency of DNA catabolism in the bloodstream,62 low transfection efficiency of plasmids in nonwounded tissues,63 stability and flexibility for compatibility with sustained delivery systems, and relative simplicity and low cost of manufacturing plasmid DNA.64 The GAM is a 3-dimensional matrix that holds DNA in situ until wound-healing fibroblasts migrate into the scaffold. Use of GAM allows for physical targeting of repair fibroblasts and other cells for direct in vivo plasmid gene transfer65 and sustained delivery of exogenous recombinant cytokines or growth factors.57,58
Gene-augmented bone repair has focused on the localized delivery of skeletal growth and differentiation factors, including bone morphogenetic proteins and the hPTH1-34 gene. The study reported here focused on the use of an hPTH1-34 GAM. Investigators used this formulation in a bovine type I collagen sponge and found that GAM combined with hPTH1-34 plasmid DNA promotes bone formation in rats and dogs with fractured tibiae.

Materials and Methods

Animals—Eight clinically normal adult horses that ranged from 3 to 15 years of age were selected for use in the study. Horses were selected on the basis of normal results for clinical, lameness, and radiographic examinations. Metacarpophalangeal (MCP) joints were deemed normal on the basis of results of lameness examinations, palpation, and radiography. All procedures were approved by an institutional laboratory animal care and use committee.

Experimental procedure—Each horse underwent surgery to create defects in both forelimbs. In each forelimb, a cortical bone defect was created in the cortex of metacarpal III and an articular defect was created in the osteochondral surface of MC III. One forelimb of each horse was randomly assigned to serve as untreated (control) defects, whereas the defects in the other forelimb were treated by use of hPTH1-34 GAM. Each horse was administered phenylbutazone (2 g, IV), gentamicin (6.6 mg/kg, IV), and procaine penicillin (4,500 IU/kg, IM) before surgery. Horses were sedated by administration of xylazine hydrochloride (1 mg/kg, IV). Anesthesia was induced by administration of thiopental (5 mg/kg, IV) and maintained with halothane vaporized in oxygen in a semiclosed system.

The forelimbs were aseptically prepared for surgery. A Robert Jones bandage was placed on each forelimb. After surgery, horses were housed separately in box stalls (5.7 X 5.7 m) for approximately 6 weeks; they had ad libitum access to hay and water. The Robert Jones bandages were removed 3 weeks after surgery. Two days before week 4 and again 10 days later, each horse received an IV injection of calcine (20 mg/kg, dissolved in 2% sodium bicarbonate solution) administered via a catheter inserted in a jugular vein. Defects were flushed with sterile saline (0.9% NaCl) solution to remove all bone shards to prevent a bone graft effect.

In forelimbs treated by use of hPTH1-34 GAM, compressible collagen sponges (3 X 1.5 cm) that contained 100 mg of plasmid DNA were pressed into the defects (Figure 2). Synovial membrane and subcutaneous layers were closed with 2-0 nonabsorbable monofilament polypropylene suture. Skin over the cortical defects was closed with 2-0 nonabsorbable monofilament polypropylene suture in a simple continuous suture pattern.

A Robert Jones bandage was placed on each forelimb. For the first 3 days after surgery, horses were examined daily for signs of pain and inflammation. All horses were administered phenylbutazone paste (2 g, PO, q 24 h) for the first 3 days after surgery. Horses were evaluated daily during the first week after surgery for signs of infection or lameness (rectal temperature, heart rate, respiratory rate, and amount of time in recumbency).

After surgery, horses were housed separately in box stalls for approximately 6 weeks; they had ad libitum access to hay and water. The Robert Jones bandages were removed 3 weeks after surgery. Two days before week 4 and again 10 days later, each horse received an IV injection of calcine (20 mg/kg, dissolved in 2% sodium bicarbonate solution) administered via a catheter inserted in a jugular vein. Defects were flushed with sterile saline (0.9% NaCl) solution to remove all bone shards to prevent a bone graft effect.

Figure 1—Sites of cortical and articular defects created in metacarpal III of both forelimbs of 8 horses. Defects (6.5 mm in diameter) were created by drilling into the middiaphysis (cortical defect) and lateral aspect of the distal condyle (articular defect).
Horses were turned out into a paddock on week 5 or 6; they remained in the paddock through week 12. In weeks 11 and 12, each horse received an injection of oxytetracycline hydrochloride (20 mg/kg, dissolved in 1 L of sterile saline solution) administered IV via a catheter inserted in the jugular vein. Calcine and oxytetracycline were administered systemically to enable us to histomorphometrically analyze bone healing (calcine is identified as a bright-green band under fluorescent light at a wavelength of 400 nm, whereas oxytetracycline is identified as a yellow-orange band under UV light at a wavelength of 400 nm). During week 13, horses were euthanatized by IV administration of a barbiturate.

Radiography—Radiographs were taken immediately before surgery (week 0 [baseline]) and 1, 4, 8, and 12 weeks after surgery. Radiographs (cranial dorsopalmar and flexed lateral views) were taken of both MC III bones and both MCP joints. Radiographs were obtained by use of long plates (43.18 x 17.78 cm) and standardized focal distance and technique settings. Radiographic assessment included scores for healing of cortical MC III and articular MC III defects. Healing scores were subjectively assigned by 1 investigator (ALB) for radiographs taken at weeks 1, 4, 8, and 12. The scoring system used was as follows: 0, defect unrecognizable; 1, defect completely unfilled; 2, defect < 25% filled; 3, defect 25% to 50% filled; 4, defect 50% to 75% filled; 5, defect > 75% filled; and 6, defect totally filled. Measurements of periosteal reaction (sum of length and thickness) were recorded.

Physical examination and joint analysis—Physical examinations were performed before surgery (baseline) and 1, 2, 3, 4, 5, 6, 8, 10, and 12 weeks after surgery. Examinations included resting rectal temperature, heart rate, respiratory rate, circumference of the MCP joint, circumference of MC III, and pain-free range of joint motion for the MCP joint. A CBC and chemical analyses were obtained at baseline and weeks 1, 2, 4, 8, and 12. Circumference of the MCP joint and MC III was recorded as the mean of 3 measurements of the flexed MCP joint. Range of pain-free motion was recorded as a percentage of 5 to 7 cm.

Lameness evaluation—Lameness evaluations were scored for trotting horses by use of the following scale: 0, lameness not perceptible; 1, normal gait while walking with a slight head nod while trotting; 2, normal gait while walking with a pronounced head nod while trotting; 3, head nod noticed while walking with a pronounced head nod while trotting; 4, severe head nod evident at all gaits and 5, lameness that caused horse to bear a minimal amount of weight while in motion or at rest or caused a horse to have a complete inability to move.

Analysis of synovial fluid—Arthrocentesis was performed immediately after surgery (baseline) and 1, 2, 4, 8, and 12 weeks after surgery. Samples of synovial fluid (3 mL) were obtained and placed in EDTA-containing collection tubes and submitted to our veterinary medical clinical pathology laboratory for subjective analysis of color, clarity, and mucin-forming clots and objective assessment of WBC counts, percentage of neutrophils, and total protein content.

Collection and processing of specimens—Immediately after the horses were euthanatized, forelimbs were harvested intact to enable us to analyze mineralization of the soft tissues by use of axial screening computed tomography (CT) at 5-mm intervals. After completion of CT, the MCP joint was incised and articular cartilage defects or score lines recorded. Synovium specimens were harvested from the MCP palmar recess and an area dorsal to the defect. Specimens were fixed in neutral-buffered 10% formaldehyde until processed. Sof tissues were removed and cortical MC III and articular defects grossly inspected. A hand saw was used to cut bone blocks that contained each defect, and the bone blocks were submitted for quantitative CT. After completion of CT, bone blocks were fixed in neutral-buffered 10% formaldehyde until processed for histomorphometric analysis.

Quantitative CT analysis—Three central 1-mm transverse slices were used to calculate the total amount of bone and density of bone in each defect. Each slice was standardized for x-ray attenuation differences for density measurements by use of potassium phosphate standards. After standardization, a calculation was performed to convert a potassium phosphate region of interest (ROI) to an ash density. Tracings of the ROI were made for the original defect, bone within the defect, and periosteal new bone. Calculations were made for bone density and bone volume for cortical and articular bone defects. Total bone volume index was calculated for cortical bone defects as the sum of the periosteal bone volume and bone volume in the defect.

Histologic preparation and evaluation of synovial membrane—Specimens of synovial membrane used for histologic examination were embedded in paraffin, sectioned (thickness of 5 to 7 µm), and stained with H&E. Dorsal and palmar synovial samples from control forelimbs and forelimbs with defects treated by use of hPTH(1-34)GAM were scored for inflammation (measured on the basis of severity of WBC infiltration and edema) and synovium cellularity (measured on the basis of the number of proliferative cells and cell necrosis). The investigator (SEW) who performed histologic scoring was not aware of the source of each specimen. Scoring for each variable was performed by use of the following scale: 0, not detected; 1, minimal; 2, mild; 3, moderate; and 4, marked. The type of inflammatory cells and a description of abnormalities were also recorded.

Histologic preparation and tissue identification of cortical and subchondral defects—Fixed bone blocks were embedded in polymethylmethacrylate and cut undecalciﬁed into sections (thickness of 200 µm) by use of a calibrated saw with a diamond cutting wheel. Sections were cut from an area near the center...
of the defect, and the surface was stained with toluidin blue and H&E to enable identification of tissue types. Tissue types were quantified within the articular MC III defect by point counting on an ocular grid at 100X magnification.

Values were expressed as a percentage for 5 general categories (fibrocortical tissue, dense fibrous tissue, bone, inflammation, and other). Bone porosity, an estimate of bone density, was quantified in the bone that filled the defect. Bone porosity was quantified by use of an automated measurement technique for the original defect area and area of bone that filled the defect in both the cortical and articular defects.

Histomorphometric analysis of calcein and oxytetracycline bone labels—Bone blocks were ground by use of a grinder to a thickness of 30 µm for histomorphometric analysis of fluorochromatic labels. Point-counting techniques (40X magnification) were used to evaluate bone label (percentage of labeled surface, percentage of mature lamellar bone, and percentage of labeled diffuse immature woven bone). Mineral apposition rate was calculated (200X magnification) in labeled trabeculae by determining the mean for 5 bone, and percentage of labeled diffuse immature woven bone).

Statistical analysis—All data were analyzed by use of a statistical software program. Objective data that were not normally distributed were logarithmically transformed prior to analysis. Nonparametric paired data that included results for histologic examination of synovium were analyzed by use of a Mann-Whitney U test. Scored radiographic data that included radiographic scores were analyzed by use of a Kruskal-Wallis test. Objective data from serial clinical results were analyzed by use of a repeated-measures ANOVA and least-significant difference post hoc tests. Single objective paired data obtained at the end of the study were analyzed by use of a paired t test; these data included results for radiographic periosteal reaction, quantitative CT, and histomorphometric analysis. Regression correlations were performed on selected variables to identify sources of concordance in data; these included correlations of quantitative CT measurements of total bone index and periosteal bone volume, with age of horse and percentage of bone that filled the defect in the untreated control limb as an estimate of native bone-forming activity. Differences were considered significant at values of \( P \leq 0.05 \).

Results

Physical examination—Data obtained from clinical evaluations were summarized (Table 1). All horses remained healthy throughout the study, as determined on the basis of physical examination variables (rectal temperature, respiration, and heart rate). All forelimbs in which defects were treated with hPTH1-34 GAM were not lame relative to the control limb at any time during the study, except for 1 horse at 1 time point (week 3) with a mild (grade 1/5) lameness. There was no significa-

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<td>joint (degrees)§</td>
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<td>52.6 ± 2.2</td>
<td>43.0 ± 1.7</td>
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<td>37.8 ± 4.1</td>
<td>43.9 ± 3.1</td>
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<td>391 ± 28.1</td>
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<td>400 ± 56.7</td>
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<td>671 ± 226</td>
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<td>fluid (%)§</td>
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<td>ND</td>
<td>ND</td>
<td>3.2 ± 0.5</td>
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</table>

*Values reported are mean ± SD for all variables, except for lameness, which is reported as median (range). †Week 0 (baseline) = Before surgery. ‡Lameness was scored on a scale of 0 to 5 (0, lameness not perceptible; 1, normal gait while walking, with a slight head nod while trotting; 2, normal gait while walking, with a pronounced head nod while trotting; 3, head nod noticed while walking, with a pronounced head nod while trotting; 4, severe head nod evident at all gaits; 5, lameness that caused horse to bear a minimal amount of weight while in motion or at rest or caused a horse to have a complete inability to move). §Values for the variable differed significantly (\( P < 0.05 \)) with time irrespective of treatment group. ND = Not determined.
significant difference between control forelimbs and forelimbs treated by use of hPTH₁-₃₄ GAM for MCP joint circumference, pain-free range of joint motion, or MC III circumference; however, values for all variables changed significantly with time. Joint circumference increased in both groups and peaked at week 4. Range of pain-free motion decreased significantly (P < 0.001) by week 2 in both groups and then gradually decreased until week 12. However, the range of pain-free motion at that time (ie, end of the study) was still significantly (P = 0.011) less than that immediately after surgery. Circumference of MC III increased after surgery and remained significantly greater, compared with the circumference immediately before surgery, until week 12. The WBC counts in synovial fluid did not differ significantly between control forelimbs and forelimbs treated by use of hPTH₁-₃₄ GAM. Furthermore, WBC counts in synovial fluid did not differ significantly over time. All mean values were < 1,000 WBCs/µL, which was within the reference range for horses established for the clinical pathology laboratory. Percentage of neutrophils and total protein content in synovial fluid did not differ significantly between control forelimbs and forelimbs treated by use of hPTH₁-₃₄ GAM; however, these values did increase significantly after surgery. Most values for the percentage of neutrophils and all mean values were < 25%, which is a typical distribution for neutrophil percentage in synovial fluid of horses. Total protein content increased after surgery, although most values were < 3.0 g/dL, which represented a mild increase over the reference range (< 2.5 g/dL) established by the clinical pathology laboratory.

Gross evaluation and crude radiographic evaluation—Mineralization of soft tissues was not detected by use of quantitative CT. Gross inspection of the cortical defects in MC III revealed that 6 of 8 treated defects and 3 of 8 control defects appeared filled with bone. Examination of cross sections revealed that most

Table 2—Mean ± SD values for quantitative computed tomography measurements of 8 horses with cortical and articular defects in metacarpal III that were untreated (control defects) or treated by use of hPTH₁-₃₄ GAM.

<table>
<thead>
<tr>
<th>Defect Variable</th>
<th>Control</th>
<th>PTH₁-₃₄GAM</th>
<th>P*</th>
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<tr>
<td>Cortical Bone volume (%)†</td>
<td>74.0 ± 21.8</td>
<td>73.0 ± 19.8</td>
<td>0.430</td>
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<tr>
<td>Periosteal bone volume (mm³)</td>
<td>9.38 ± 12.57</td>
<td>27.08 ± 38.30</td>
<td>0.070</td>
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<td>Total bone volume index (%)‡</td>
<td>1.88 ± 0.17</td>
<td>1.98 ± 0.20</td>
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<td>Whole defect density (mg/mm³)§</td>
<td>951.9 ± 274.2</td>
<td>840.5 ± 274.6</td>
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<tr>
<td>Bone filling density (mg/mm³)</td>
<td></td>
<td>1,154 ± 153</td>
<td>1,028 ± 179</td>
</tr>
<tr>
<td>Periosteal bone density (mg/mm³)</td>
<td>414.1 ± 452.6</td>
<td>452.6 ± 390.8</td>
<td>0.399</td>
</tr>
<tr>
<td>Articular Bone volume (%)†</td>
<td>59.0 ± 15.4</td>
<td>34.0 ± 1.9</td>
<td>0.010</td>
</tr>
<tr>
<td>Whole defect density (mg/mm³)§</td>
<td>818.0 ± 220.4</td>
<td>479.6 ± 175.5</td>
<td>0.020</td>
</tr>
<tr>
<td>Bone filling density (mg/mm³)</td>
<td></td>
<td>982.3 ± 143.2</td>
<td>919.9 ± 131.5</td>
</tr>
</tbody>
</table>

*Values were considered significant at P ≤ 0.05. †Value reported as percentage of the original defect. ‡Total bone volume index is the sum of bone volume filling the defect and periosteal bone volume. §Tracing of the whole area within the defect, including bone and nonbone areas. A lower density correlates with less bone and more tissue area within these defects. ||Density exclusively for bone within the defect.

Figure 3—Quantitative computed tomography of transverse central slices of cortical (A) and subchondral (B) bone defects in metacarpal III from a representative control limb and a limb treated by use of hPTH₁-₃₄ GAM. The samples were obtained when the horse was euthanatized 13 weeks after creation of the defects. Notice the cortical defect treated by use of hPTH₁-₃₄ GAM had an increase in total bone volume, primarily periosteal bone, compared with the control defect.

Figure 4—Quantitative computed tomography of a transverse central slice of the control and hPTH₁-₃₄ GAM-treated cortical defects in metacarpal III from a horse that did not respond to treatment with PTH₁-₃₄ GAM. Notice the minimal bone healing in the control cortical defect, which is indicative of a lower rate of bone formation in this horse.
have a lower or no response to hPTH1-34 GAM for bone-forming volume in untreated defects did not correlate between the amount of bone filling the defect, amount of periosteal bone, or effect of hPTH1-34 GAM on defect or periosteal bone volume and age or sex of horse. However, horses with lower values of hPTH1-34 GAM on defect or periosteal bone volume and age or sex of horse. However, horses with lower values for bone-forming volume in untreated defects did have a lower or no response to hPTH1-34 GAM on defect or periosteal bone volume and age or sex of horse. However, horses with lower values for bone-forming volume in untreated defects did have a lower or no response to hPTH1-34 GAM on defect or periosteal bone volume and age or sex of horse. However, horses with lower values for bone-forming volume in untreated defects did have a lower or no response to hPTH1-34 GAM on defect or periosteal bone volume and age or sex of horse. However, horses with lower values for bone-forming volume in untreated defects did have a lower or no response. Radiographic scores increased with time, but there was not a significant difference in scores between treated and control cortical or articular defects. The periosteal reaction (sum of the length and depth of new periosteal bone) was greater, but not significantly (P = 0.07) different amount of bone filling the defect had significantly more bone that filled the defect (BFD). Void = Empty drill hole. H&E stain. Bar = 0.1 mm.

defects treated by use of hPTH1-34 GAM visually appeared to have more bone formed over the defect.

Histologic examination of synovial membranes—The synovium appeared grossly normal in all joints. Synovium harvested from the dorsal aspect of the MCP joint over the defect did not have significant differences in scores for synovial inflammation or synovial necrosis among treated joints or when compared with scores for control joints.

Quantitative CT—Data obtained from quantitative CT measurements were summarized (Table 2). Quantitative CT detected a significantly (P = 0.05) greater amount of total bone volume and a greater but not significantly (P = 0.07) different amount of periosteal bone around cortical defects treated by use of hPTH1-34 GAM (Figure 3). There was no significant difference between treated and control limbs in the amount of bone that filled into a defect because defects were mostly healed. There was no significant correlation between the amount of bone filling the defect, amount of periosteal bone, or effect of hPTH1-34 GAM on defect or periosteal bone volume and age or sex of horse. However, horses with lower values for bone-forming volume in untreated defects did have a lower or no response to hPTH1-34 GAM on defect or periosteal bone volume and age or sex of horse. However, horses with lower values for bone-forming volume in untreated defects did have a lower or no response to hPTH1-34 GAM on defect or periosteal bone volume and age or sex of horse. However, horses with lower values for bone-forming volume in untreated defects did have a lower or no response to hPTH1-34 GAM on defect or periosteal bone volume and age or sex of horse. However, horses with lower values for bone-forming volume in untreated defects did have a lower or no response to hPTH1-34 GAM on defect or periosteal bone volume and age or sex of horse.

Quantitative histomorphometric analysis—Analysis of histomorphometric data confirmed the quantitative CT results and revealed no significant (P = 0.41) difference between treated and control limbs for the area of bone filling the cortical defect, as measured by computerized automated image analysis. The area of the original defect was clearly visible in all limbs and was mostly filled with woven bone. There was no evidence of inflammation or cyst formation. There was no significant (P = 0.21) difference in the bone porosity between control defects and defects treated by use of hPTH1-34 GAM (Table 3). Cortical defects consisted of woven bone.

Computerized image analysis of articular bone volume also corroborated CT results and had less, but not significantly (P = 0.09), area of bone filling the defect in treated (50%) defects, compared with the value for control defects (73%). Bone porosity was not significantly different between treated (mean, 24.1%) and control (mean, 11.4%) defects for the bone that filled articular defects. Healing bone appeared morphologically normal in both treated and control articular defects.

Cortical defects had little nonbone tissue, which consisted of fibrous tissue in the central core (Table 3). Articular defects treated by use of hPTH1-34 GAM had less bone and cartilage and greater amounts of loose

<table>
<thead>
<tr>
<th>Defect</th>
<th>Group</th>
<th>Bone tissue</th>
<th>Nonbone tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bone</td>
<td>Porosity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bone</td>
<td>Porosity</td>
</tr>
<tr>
<td>Cortical</td>
<td>Control</td>
<td>90.0 ± 0.8</td>
<td>10.0 ± 0.8</td>
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<tr>
<td></td>
<td>hPTH1-34 GAM</td>
<td>88.6 ± 0.7</td>
<td>12.0 ± 0.7</td>
</tr>
<tr>
<td>Articular</td>
<td>Control</td>
<td>88.6 ± 4.2</td>
<td>11.4 ± 4.2</td>
</tr>
<tr>
<td></td>
<td>hPTH1-34 GAM</td>
<td>75.9 ± 10.1</td>
<td>24.1 ± 10.1</td>
</tr>
</tbody>
</table>

* †Values differ significantly (*P = 0.03; †P = 0.015).
FT = Fibrous tissue. FC = Fibrocartilage. HC = Hyaline cartilage. I = Inflammation. Other = Loose mesenchymal tissue, remnants of the collagen sponge, and tissue void.

(Figure 4). Indeed, 3 horses that had values > 1 SD from the mean volume of the amount of bone filling the defect had significantly (P = 0.029) lower amounts of total and periosteal bone that formed in the treated defects, compared with values for the other 5 treated horses. Forelimbs treated by use of hPTH1-34 GAM had a significantly (P = 0.003) lower density for the bone that filled the defect. Density of the periosteal new bone did not differ significantly (P = 0.38) between control defects and defects treated by use of hPTH1-34 GAM.

Quantitative CT measured in articular defects treated by use of hPTH1-34 GAM had significantly (P = 0.01) less bone filling the defects (Figure 3). This resulted in a significantly (P = 0.02) lower density of total tissue filling the original defect. There was no significant difference in density of the bone that was within the articular defects.

Quantitative histomorphometric analysis—Analysis of histomorphometric data confirmed the quantitative CT results and revealed no significant (P = 0.41) difference between treated and control limbs for the area of bone filling the cortical defect, as measured by computerized automated image analysis. The area of the original defect was clearly visible in all limbs and was mostly filled with woven bone. There was no evidence of inflammation or cyst formation. There was no significant (P = 0.21) difference in the bone porosity between control defects and defects treated by use of hPTH1-34 GAM (Table 3). Cortical defects consisted of woven bone.

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Cortical defects had little nonbone tissue, which consisted of fibrous tissue in the central core (Table 3). Articular defects treated by use of hPTH1-34 GAM had less bone and cartilage and greater amounts of loose
Table 4—Histomorphic analysis for 8 horses for fluorochromat-
ic bone labeling by use of calcein administered approximately 4
and 5 weeks after creation of cortical and articular defects in
metacarpal III that were untreated (control defects) or treated
by use of hPTH$_{1-34}$ GAM.

<table>
<thead>
<tr>
<th>Defect</th>
<th>Variable</th>
<th>Control</th>
<th>hPTH$_{1-34}$ GAM</th>
<th>$P^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortical</td>
<td>Total labeled bone (%)</td>
<td>26.6</td>
<td>17.7</td>
<td>0.020</td>
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<tr>
<td></td>
<td>Amorphous label (%)†</td>
<td>64.9</td>
<td>81.3</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>Linear label (%)‡</td>
<td>35.1</td>
<td>18.7</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>Mineral apposition rate (µm/d)</td>
<td>1.59</td>
<td>1.79</td>
<td>0.070</td>
</tr>
<tr>
<td>Articular</td>
<td>Total labeled bone (%)</td>
<td>46.2</td>
<td>21.8</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>Amorphous label (%)†</td>
<td>78.2</td>
<td>67.1</td>
<td>0.100</td>
</tr>
<tr>
<td></td>
<td>Linear label (%)‡</td>
<td>21.8</td>
<td>18.6</td>
<td>0.370</td>
</tr>
<tr>
<td></td>
<td>Mineral apposition rate (µm/d)</td>
<td>1.76</td>
<td>1.69</td>
<td>0.440</td>
</tr>
</tbody>
</table>

*Values were considered significant at $P \leq 0.05$. †Represents woven bone. ‡Represents mature lamellar bone.

Fluorescence histomorphometric analysis and mineral apposition rate—Data for fluorescent histomorphometric analysis were summarized (Table 4). Analysis of calcein labeling performed twice after surgery revealed significantly less labeled bone in the defects treated by use of hPTH$_{1-34}$ GAM, compared with control defects, for both cortical ($P = 0.028$) and articular ($P = 0.005$) defects (Figure 6). Cortical defects treated by use of hPTH$_{1-34}$ GAM had significantly ($P = 0.007$) more labeled woven bone, compared with the amount of labeled lamellar bone in control defects (Figure 7). In articular defects, little bone was evident in treated defects, and differences could not be detected in percentages of labeled bone between treated and control defects. Mineral apposition rate was slightly but not significantly ($P = 0.07$) greater in the treated cortical defects, compared with the value for the articular defects.

Oxytetracycline label was less intense and a lesser total amount than for the calcein label. Oxytetracycline label was evident in lamellar and woven bone in the defects and periosteal bone of cortical defects. There was no calcein label in periosteal bone, which indicated that periosteal bone formed more than 5 weeks after the start of healing. Oxytetracycline label was only evident in lamellar bone of maturing trabeculae within the central aspects of defects, which indicated that healing began at the edges of the defects and progressed toward the center of the defects.

Discussion

To our knowledge, the study reported here is the first in which horses have been used to document that a construct of hPTH$_{1-34}$ GAM can promote bone formation, specifically periosteal bone formation (Figure 3). Most horses responded with a robust periosteal reaction that was obvious during standard clinical examination (ie, palpation, gross observation, and radiographic evaluation). Interestingly, a few horses did not respond to hPTH$_{1-34}$ GAM; however, bone formation was significantly ($P = 0.002$) less in the control limbs of those horses, which indicated a general reduction in bone formation in those horses (Figure 4). Correspondingly, hPTH$_{1-34}$ GAM was more potently osteoinductive in cortical defects, particularly periosteal new bone, in horses that had greater bone volume in the untreated defects. Age of the horse was not a factor correlated with response.

An association between poor response to other bone forming genes (eg, bone morphogenetic protein) and low bone formation has been reported in nonhuman primates$^*$ and rats$^5$ and may be related to less mesenchymal tissue and void space than control defects (Figure 5). Inflammation was not a prominent feature of control or treated defects. Areas of loose mesenchymal tissue contained scattered areas of birefringence when examined under polarized light; this was interpreted to represent residual pieces of the collagen sponge.
activity of gene promoters in those animals that cannot be overridden by downstream regulators of bone formation, such as PTH and bone morphogenetic protein. It is also possible that hPTH1-34 GAM may have migrated through the endosteal surface of the defect and into the marrow cavity, which resulted in minimal to no effects in 3 horses; however, this would not explain the association with low bone-forming ability. Importantly, the increase in total bone volume seen in the other horses was sufficient to provide significant differences necessary to conclude that there was an influence of the hPTH construct on bone induction. The periosteal location of the bone response may have been attributable to migration of the construct toward the periosteal surface of the cortex, particularly as the defect filled with tissue, or to the fact that periosteal cells are particularly receptive to gene transduction or PTH.

Clinical application of the hPTH1-34 GAM construct in cortical bone will require additional studies. Use of less collagen per unit area and a greater plasmid complementary DNA-to-collagen ratio may ameliorate the delay in bone formation. For cortical fractures and stress fractures in the dorsal aspect of the cortex of MC III, periosteal bone formation can be the major source of healing bone for stabilization of the fracture. However, if the construct delays the onset of bone formation and bone is less dense, this could be a critical impediment to a successful outcome.

Although bone formation around defects treated by use of hPTH1-34 GAM was greater by 13 weeks after surgery, compared with formation around control defects, histomorphometric analysis of the calcein label within the cortical defects documented less bone formation and a less mature bone than for control defects (Figure 6). This result, in conjunction with the fact that labeled bone in the control defects was more mature lamellar bone, indicated an earlier start for bone formation in control defects (Figure 7). Histologic examination did not reveal differences in bone resorption. Osteoclasts and resorption lacunae were uncommon, and the numbers of these were subjectively similar in treated and control defects, which indicated that hPTH1-34 GAM probably delayed early bone formation in treated cortical defects. Osteoinduction by hPTH1-34 GAM overcame this effect by 13 weeks. Resorption of the collagen sponges is suspected to have impaired early bone formation. Other studies of bones that involved the use of collagen carriers did not use empty (ie, not filled with the collagen matrix) control defects; therefore, this effect would not have been detectable. Importantly, our goal was to evaluate the hPTH1-34 GAM construct as a potential therapeutic tool. Therefore, it was critical to enhance healing from that seen for the natural state, rather than compared with that for a collagen-implanted defect.

The hPTH1-34 GAM construct impeded bone formation in the subchondral (articular) defects that persisted for the 13-week study (Figure 3). Increased intrasseous pressure from increased intra-articular pressure can cause lysis of subchondral bone and clinical persistence of defects. Microfractures of the articular subchondral bone in areas in which there is synovial fluid have been documented to cause subchondral bone cysts. These cysts are believed to result from synovial fluid being forced under pressure into the underlying bone through defects in the cartilage, which causes subsequent dissolution of the bone.

The major types of tissue that filled treated defects included a predominance of fibrous tissue (articular defects) and loose mesenchymal tissue (articular defects), compared with tissue types that filled control defects. Observation of these articular sections by use of polarized light revealed a linear network that was interpreted as representing collagen remnants from the collagen sponge. Excessive size of the sponge (to allow dense packing) and slower absorption of collagen in the joint environment may have contributed to the delay in bone formation. Additionally, the lack of periosteal cells in subchondral bone would predict a poor response to hPTH1-34 GAM in the joint environment, considering the predominantly periosteal bone response to hPTH1-34 GAM in the cortex.

Although bone formation was suppressed in articular defects treated by use of hPTH1-34 GAM, we found that this construct was biocompatible with joint tissues. Histologic examination of synovium did not reveal differences in variables used to evaluate inflammation or histologic changes of synovial membranes. Gross evaluation of the joints and CT scans of the soft tissues of the joint around a treated defect did not reveal evidence of thickened soft tissues or soft tissue mineralization. For treated defects, bone did not form in abnormal locations around defects, on the endosteal surface, or in bone marrow spaces, which indicated that the sponge most likely did not migrate out of the defect and was compatible with the joint environment. Clinical examinations and results of hematologic analysis supported clinical safety for use of hPTH1-34 GAM in and near joints.

The construct of hPTH1-34 GAM was osteoinductive in diaphyseal periosteal bone and potentially localized its transgene expression or response in periosteal cells. The construct of hPTH1-34 GAM was detrimental to bone healing when placed into bone through an articular defect. This construct was biocompatible for use in or near joints; shortening the collagen hPTH1-34 contact articular surfaces, it is unlikely to cause short-term adverse effects. The potential benefit of this gene therapy to stimulate periosteal bone formation requires additional investigation to determine whether it could be of benefit in animals with fractures of cortical bones with periosteum.

3p-Mat-1 National Gene Vector Laboratory hPTH1-34 in bovine type I collagen sponge. Selective Genetics Inc, San Diego Calif.
4Equi-Phar phenylbutazone injection 20%, Vedco Inc, St Joseph, Mo.
5Gentamicin sulfate solution, Butler Co, Columbus, Ohio.
6Aquacllin, Vedco Inc, St Joseph, Mo.
7Sedazine, Fort Dodge Animal Health, Fort Dodge, Iowa.
8Sodium Pentothal, Abbott Laboratories, North Chicago, Ill.
9Halothane, Halocarbon Laboratories, River Edge, NJ.
0.9% sodium chloride solution, Baxter Healthcare Corp, Deerfield, Ill.
1Vicyr, Ethicon, Somerville, NJ.
2Surgilene, Sherwood, Davrge, Geck, St Louis, Mo.
3Phenylozone, Schering-Plough Animal Health, Union, NJ.
References


