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Recombinant Human Bone Morphogenetic Protein-2 Enhances Osteotomy Healing in Glucocorticoid-Treated Rabbits*

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ABSTRACT

The objectives of this study were to evaluate the effect of chronic prednisolone treatment on osteotomy healing in rabbits and to determine whether recombinant human bone morphogenetic protein-2 (rhBMP-2) would enhance healing in the presence of chronic glucocorticoid therapy. Forty-nine skeletally mature, male rabbits were injected with either prednisolone ($n = 26$; 0.35 mg/kg per day, three times a week) or saline ($n = 23$). After a 6-week pretreatment period, bilateral ulnar osteotomies were created surgically. One osteotomy was treated with rhBMP-2 (0.2 mg/ml of rhBMP-2, 40 μg of rhBMP-2 total) delivered on an absorbable collage sponge (ACS), whereas the contralateral osteotomy remained untreated. Prednisolone or saline treatment was continued until the rabbits were killed either 6 weeks or 8 weeks after creation of the osteotomy. Osteotomy healing was evaluated by radiography, peripheral quantitative computed tomography (pQCT), torsional biomechanics, and undecalcified histology. Because we observed similar responses to both prednisolone and rhBMP-2/ACS treatment in the 6-week and 8-week cohorts, the results from these time points were combined. Serum osteocalcin and vertebral trabecular bone density were lower in the prednisolone-treated rabbits. Prednisolone treatment dramatically inhibited osteotomy healing. In the untreated ulnas, callus area and torsional strength were 25% and 55% less, respectively, in the prednisolone-treated rabbits than in the saline group ($p < 0.001$ for both). rhBMP-2/ACS enhanced healing in both the prednisolone- and the saline-treated groups, although the effect was larger in the prednisolone-treated rabbits. In the prednisolone-treated rabbits, callus area and torsional strength were 40% and 165% greater ($p < 0.001$ for both), respectively, in osteotomies treated with rhBMP-2/ACS compared with the contralateral, untreated osteotomies. Histological evaluation confirmed that osteotomy healing was inhibited by prednisolone and accelerated by rhBMP-2/ACS. In summary, a single application of rhBMP-2/ACS counteracted the inhibition of osteotomy healing caused by prednisolone exposure. These results suggest that rhBMP-2/ACS may be a useful treatment for enhancing fracture healing in patients who are undergoing chronic glucocorticoid therapy. (J Bone Miner Res 2002;17:301–310)

Key words: fracture healing, glucocorticoid, prednisolone, recombinant human bone morphogenetic protein-2, rabbit, biomechanics, osteotomy

INTRODUCTION

GLUCOCORTICOID THERAPY is prescribed widely for its anti-inflammatory and immunosuppressive effects. Although useful in the treatment of respiratory, skin, and musculoskeletal diseases, pharmacologic doses of glucocorticoids influence bone metabolism, leading to impaired bone healing and bone loss.[1–3] The majority of patients taking glucocorticoids for over 6 months develop osteoporosis and, consequently, patients on high-dose therapy have an increased risk of fracture.[2–4]

Several mechanisms, both indirect and direct, have been proposed to account for the effects of glucocorticoid therapy on bone. Glucocorticoid therapy indirectly influences bone metabolism by altering calcium homeostasis and secretion of sex hormones.[5] In particular, pharmacologic doses of

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glucocorticoids lead to reduced calcium absorption in the gut, secondary hyperparathyroidism, and decreased secretion of anabolic hormones.[6] The direct actions of glucocorticoids on bone cells lead to inhibition of bone formation, suppression of osteoblastogenesis, increased bone resorption, and enhanced apoptosis of osteoblasts and osteocytes.[7] In addition, glucocorticoids influence local production of growth factors and cytokines that may affect bone formation and resorption.[8] Taking these results all together, it appears that the net result of the endocrine and paracrine responses to glucocorticoids is inhibition of osteoblasts and activation of osteoclasts.[5]

Consistent with their detrimental effects on bone density and fracture risk, glucocorticoids also inhibit fracture healing. In the mid-1970s, Kostenszky et al.[9] used autoradiography and histology to show that administration of high-dose glucocorticoids delayed mineralization of the fracture callus in a dog model. More recent studies have shown that systemic prednisolone therapy impairs fracture healing in rabbits.[10,11] The proposed mechanisms of glucocorticoid action in bone that were outlined previously likely contribute to this impaired fracture healing. Specifically, the reduction in bone formation and inhibition of osteoblastogenesis seen after glucocorticoid therapy could inhibit fracture healing. Moreover, localization of the functional glucocorticoid receptor in human chondrocytes, osteoblasts, and osteocytes suggests that all stages of endochondral bone formation, including those normally involved in fracture healing, may be influenced by glucocorticoid therapy.[12]

Bone morphogenetic protein-2 (BMP-2) is a potent differentiation factor that is essential for embryonic skeletal development and for osteoblastogenesis in the adult.[13,14] In addition, BMP-2 is up-regulated during fracture healing.[15,16] The primary action of BMP-2 is to induce the differentiation of mesenchymal precursor cells into osteoblasts, although it also may play an indirect role in the chemotactic and proliferative behavior of these cells.[17] In several animal models, exogenous application of recombinant human BMP-2 (rhBMP-2) has been shown to promote healing of critical-sized, long bone defects[18–20] and to accelerate healing of diaphyseal fractures.[21–23]

Despite the important clinical ramifications, few studies have investigated the potential interaction between inhibition of bone formation by glucocorticoid therapy and stimulation of bone formation by rhBMP-2 in vivo. Although in vitro studies have shown that physiological levels of glucocorticoids potentiate the proliferative effects of rhBMP-2[24,25] and that the inhibition of osteoblastogenesis induced by pharmacologic levels of glucocorticoids may be counteracted by rhBMP-2,[26] it is not known whether glucocorticoids will inhibit the stimulatory effects of rhBMP-2 on bone formation in vivo. Therefore, this study was designed to investigate this potential interaction between glucocorticoid therapy and rhBMP-2 in vivo. We used a rabbit ulnar osteotomy model to evaluate whether fracture healing is inhibited by chronic glucocorticoid therapy and if so, whether rhBMP-2 can overcome this inhibition and enhance healing.

MATERIALS AND METHODS

Study design

Forty-nine skeletally mature, male New Zealand White rabbits (>8 months old, 3.5–4.0 kg) were assigned to two groups: prednisolone or control (saline). Bilateral, midulnar osteotomies were created surgically in all rabbits. One limb was treated with rhBMP-2/absorbable collage sponge (ACS; total dose = 40 μg of rhBMP-2), whereas the contralateral limb remained untreated. To simulate chronic glucocorticoid therapy, the rabbits received systemic prednisolone (or saline) therapy for 6 weeks before creation of the osteotomy as well as throughout the healing period. Rabbits in the prednisolone group received subcutaneous injections of prednisolone three times a week (0.35 mg/kg per day, totaling 1.05 mg/kg per week; Steris Laboratories, Phoenix, AZ, USA), whereas those in the saline group received subcutaneous injections of normal saline according to the same schedule. This prednisolone dosing regimen was shown previously to induce bone loss and inhibit fracture healing in rabbits and to be tolerable for long-term administration.[10,11,27] Rabbits were assigned randomly to be killed at either 6 weeks (13 rabbits, prednisolone; 12 rabbits, saline) or 8 weeks (13 rabbits, prednisolone; 11 rabbits, saline) after creation of the osteotomies. These time points were selected based on a previous study using the same model in which the osteotomies were healed at 6 weeks.[23] Thus, the 8-week time point was chosen to account for the potential of delayed healing in the prednisolone group. The rabbits were monitored daily to assess general health and neurological function and were weighed weekly to determine prednisolone (and saline) injection volumes. The rabbits were housed individually and fed standard rabbit chow and water ad libitum. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the Genetics Institute and all procedures were carried out according to Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines.

Surgical procedure and general animal care

Bilateral, midulnar osteotomies were created surgically in all rabbits. The rabbits received injections of analgesics (Buprenex, 0.3 mg/kg twice a day [b.i.d.]; Recitt & Colman Pharmaceuticals, Inc., Richmond, VA, USA) and antibiotics (Monocid, 25 mg/kg once a day [s.i.d.]; SmithKline Beednam, Philadelphia, PA, USA) before surgery and for 2 days after surgery. Before surgery, rabbits were anesthetized by intramuscular injections of ketamine (35 mg/kg) and xylazine (5 mg/kg). Both forelimbs were shaved and prepped with chlorhexidine scrub, 70% ethanol, and chlorhexidine solution. After sedation and surgical preparation, the rabbit was placed in a supine position and general anesthesia by halothane/oxygen was administered. A posterior longitudinal incision was made over the ulna and the underlying muscles were retracted, exposing the middiaphyseal surfaces of both the ulna and the radius. Care was taken to minimize damage to the periosteum. Osteotomies were created approximately 45–50 mm distal to the olecranon process using a high-speed oscillating saw (0.5- to 1-mm blade width defect). During the osteotomy procedure, the site was

irrigated continuously with saline containing 0.2 mg/ml gentamicin sulfate to minimize thermal damage. In each rabbit, one ulna was treated with rhBMP-2 and the contralateral ulna was left untreated. Previously, we have shown that healing of osteotomies treated with an ACS that has been soak-loaded with buffer is equivalent radiographically, histologically, and biomechanically to untreated osteotomies.[23] Thus, in this study, we chose to use an untreated osteotomy for the control limb.

The rhBMP-2 (200 μl of 0.2 mg/ml of rhBMP-2) was applied to an 8 mm $\times$ 20 mm strip of ACS (Integra Life Sciences Corp., Plainsboro, NJ, USA) at least 30 minutes before implantation. Then, the rhBMP-2/ACS implant was placed on the palmar aspect of the ulna and wrapped circumferentially around the lateral and part of the dorsal aspect of the ulna. The medial aspect of the ulna could not be reached because of the position of the radius. Therefore, the rhBMP-2/ACS implant surrounded approximately two-thirds of the ulna. After ensuring adequate hemostasis, the wound was closed in layers. The rabbit was taken into recovery, placed in lateral recumbency, and monitored until the righting reflex returned. All rabbits were ambulatory after surgery. Because the osteotomy was stabilized by the radius, no casts or splints were used. Fluorochrome labels were administered 10 days after surgery (tetracycline, 25 mg/kg) and 13 days and 3 days before death (calcein, 5 mg/kg). Ulnas from 10 rabbits per group were designated for biomechanical testing, and 2 representative ulnas from each group were selected for histological evaluation. To choose the ulna for histological evaluation, we reviewed the final set of in vivo radiographs and ranked the rabbits in each group according to the extent of healing. The two rabbits that ranked in the middle of each group with respect to healing were chosen for histological evaluation. This approach ensured that, for our qualitative histological evaluation, we chose ulnas that represented the average healing of the group. After euthanasia, the rabbit forelimbs were cleared carefully of excess tissue and stored frozen at -20°C for biomechanical testing or fixed in 70% ethanol for histology.

Evaluation of the systemic effect of prednisolone treatment

To assess the systemic effect of prednisolone treatment, we monitored bone formation via serum osteocalcin measurements. Serum samples were obtained at 0, 3, 6 (osteotomy surgery), and 12 weeks after the initiation of prednisolone or saline treatment. Serum osteocalcin was determined using a competitive immunoassay that binds osteocalcin in serum using a monoclonal antiosteocalcin antibody (NovoCalcin; Metrabiosystems, Mountain View, CA, USA).

In addition, we performed static and dynamic histomorphometry of vertebral trabecular bone. At death, the sixth lumbar vertebral body was dissected free from surrounding soft tissues and placed in 70% ethanol for fixation. Undecalcified specimens were processed through alcohol gradients, embedded in methylmethacrylate (MMA), and sectioned in the midsagittal plane.[28] Thin sections (8 μm) were either stained using a modified von Kossa[28] for static histomorphometric analysis or left unstained for dynamic histomorphometric analyses. Dynamic histomorphometry measurements were performed for the rabbits killed at the 6-week time point only. Trabecular perimeter, trabecular area, and total area were measured using KSS stereology software (KSS, Salt Lake City, UT, USA). Static parameters (trabecular bone volume [BV/TV, %], trabecular thickness [Tb.Th, μm], trabecular separation [Tb.Sp, μm], and trabecular number [Tb.N]) were calculated based on a plate model.[29] Label length and double label width were measured using Osteomeasure software (Scimeasure Analytical Systems, Inc., Atlanta, GA, USA) and dynamic indices (mineral apposition rate [MAR, μm/day], mineralizing surface [MS, %], and bone formation rate [BFR, %/year]) were calculated according to methods described previously.[29] The individual performing the histological evaluation was blinded to the study design and treatment groups.

Evaluation of osteotomy healing

Osteotomy healing was evaluated in vivo using anteroposterior and lateral radiographs. Postmortem assessments included peripheral quantitative computed tomography (pQCT) to assess the geometry and density of the mineralized callus, torsional biomechanics to assess the mechanical properties of the healing osteotomy, and undecalcified histology.

Anteroposterior and lateral radiographs (40 kVp, 200 mA, 0.2 s; Kodak Ektascan M film; Kodak, Rochester, NY, USA) were taken immediately postoperatively and weekly thereafter. The radiographs were evaluated by an independent reader who was blinded to the study design and treatment groups. We recorded the onset of callus mineralization and the week that each of the four cortices first appeared bridged with bone. When three of four cortices were bridged, the ulna was designated as "radiographically bridged." An ulna was designated as having achieved "radiographic union" when all four cortices were bridged, there was a radiopaque callus, and the osteotomy line was no longer evident.

To determine the density (mg/cm^3), bone mineral content (g), and area (mm^2) of the mineralized callus, the excised limbs were scanned using pQCT (XCT3000; Stratec, Pforzheim, Germany). For each forelimb, we acquired five contiguous slices through the fracture callus, perpendicular to the diaphyseal axis. The slices were 2.2 mm thick, 2 mm apart, and had an in-plane pixel size of 0.2 $\times$ 0.2 mm. The three slices surrounding the osteotomy, including a middle slice that was closest to the osteotomy, were analyzed. To analyze each slice, a region of interest was drawn around the ulna and its callus, excluding the radius. The first analysis used a low bone density threshold (375 mg/cm^3) to measure the properties of the ulna and callus. Then, a second analysis was performed using a high threshold (750 mg/cm^3) to measure the properties of the ulna only. Combining the data from these two analyses yielded the area, mineral content, and density of the fracture callus itself. Average values from the three slices are reported.

Ulnas from 10 rabbits per group were tested biomechanically using a servohydraulics materials testing system (model 8500; Instron Corp., Canton, MA, USA). The fore-

limbs were cleared carefully of soft tissue and then embedded for testing in square molds using poly-MMA (PMMA). Once embedded, a high-speed saw was used to cut through the radius so that it could be gently pried away from the ulna. Care was taken to not disturb the callus or ulna. After embedding, the ulnas were tested to failure in torsion at a constant loading rate (1.5°/s) and torque and rotation data were recorded at 50 Hz. Failure torque (N-m), torsional stiffness (N-m/deg), and energy absorbed to failure (N-m/deg) were computed from the torque versus rotation data. For those specimens with no clear failure, the maximum rotation and energy-to-failure were computed for a displacement up to 35°. Faxitron radiographs of the ulnas were performed after mechanical testing and used to determine the location of the fractures according to a modification of the guidelines established by White et al.[30] Thus, the ulnas were classified as having fractured either through the original osteotomy site, through a combination of the original osteotomy site and intact host bone, or through the intact host bone only.

Based on radiographic observations, ulnas from two representative rabbits per group were excluded from biomechanical testing and were instead evaluated for callus composition, defect bridging, and cortical continuity using qualitative undecalcified histology. After fixation in 70% ethanol, the specimens were processed through alcohol gradients, embedded in MMA, and sectioned in a sagittal plane through the center of the diaphysis and region of interest. Sections (5 μm thick) were placed on slides and stained with Goldner's trichrome.

Data analysis

Standard descriptive statistics were computed for all outcome variables. Analysis of variance (ANOVA) was used to determine the effect of endpoint (6 weeks or 8 weeks). Because this analysis suggested that the effects of prednisolone and rhBMP-2/ACS treatments were similar in the 6-week and 8-week cohorts, results from the two time points were combined for all subsequent analyses, except for the qualitative histology evaluations. The systemic effect of prednisolone exposure was evaluated by comparing histomorphometry of vertebral trabecular bone using a Student's *t*-test and serum osteocalcin levels using an ANOVA with repeated measures in the prednisolone and saline groups. The effect of prednisolone treatment on osteotomy healing was evaluated by comparing properties of the untreated limbs in the prednisolone and saline groups by Student's *t*-tests (for pQCT and biomechanics) or χ^2 analyses (for radiographic union and fracture location). The effect of rhBMP-2/ACS on fracture healing was evaluated using an ANOVA with repeated measures and paired *t*-tests (for pQCT and biomechanics) as well as χ^2 analyses for assessment of radiographic union. Data are presented as means ± SD unless otherwise noted. All tests were two-tailed and differences were considered significant at $p < 0.05$. Post hoc testing was done using Fisher's protected least squares difference test. Statistical analyses were done using Statview 5.0 (SAS Institute, Inc., Cary, NC, USA) and SuperANOVA (SAS Institute, Inc.).

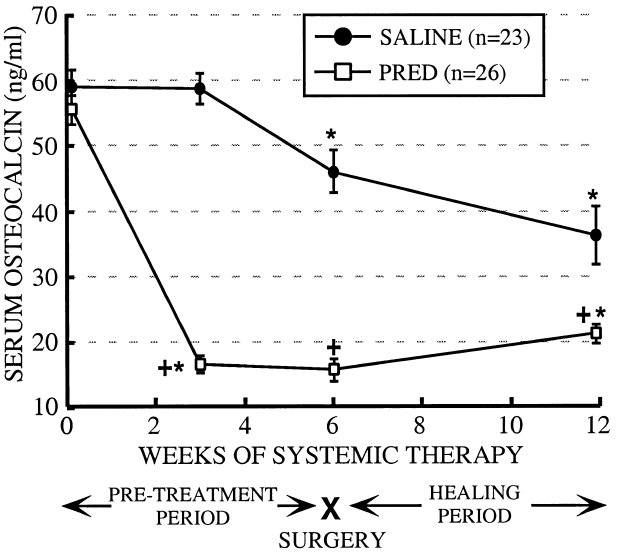

FIG. 1. Serum osteocalcin (ng/ml; mean ± SEM) in prednisolone- and saline-treated rabbits over 12 weeks of treatment. +Prednisolone < saline ($p < 0.0001$); *significant change from previous time point ($p < 0.05$).

RESULTS

The surgical procedure was well tolerated. The rabbits were fully weight bearing immediately after surgery and were freely ambulating shortly thereafter. Throughout the study, the rabbits displayed normal eating and behavior patterns. Despite this, all rabbits lost weight during the course of the study, with the majority of the weight loss (approximately 200 g) occurring the week after surgery. Over the course of the study, the average weight loss in the prednisolone-treated rabbits was greater than that in the saline-treated rabbits (390 ± 200 g vs. 220 ± 160 g; $p = 0.01$). Because rabbits are expected to lose weight after surgery and there was large intragroup variation in the weights, we consider the difference in weight loss between the prednisolone and saline groups to be a minor finding.

Effects of prednisolone on bone metabolism and vertebral trabecular bone histomorphometry

Osteocalcin levels in the prednisolone group were 42–72% lower than those in the saline group as early as 3 weeks after initiation of prednisolone treatment and continuing throughout the study ($p < 0.0001$ for all; Fig. 1). In the prednisolone group, osteocalcin levels were 62–72% below baseline levels at all time points after initiation of prednisolone treatment ($p < 0.0001$ for all). In the saline group, osteocalcin levels were 22% and 38% lower than baseline at 6 weeks and 12 weeks, respectively, after initiation of saline injections ($p < 0.0005$ for both).

Both static and dynamic histomorphometric variables from vertebral trabecular bone were consistently lower in the prednisolone group, although the differences did not always reach statistical significance (Table 1). Trabecular BV/TV was 11.5% lower ($p = 0.07$) and Tb.Th was 7.6% lower ($p = 0.03$) in the prednisolone-treated rabbits com-

TABLE 1. STATIC AND DYNAMIC HISTOMORPHOMETRY OF THE LUMBAR VERTEBRAE (MEAN ± SD)

Variable	*Prednisolone*	*Saline*
BV/TV (%)	17.7 ± 3.4	20.0 ± 5.1*
Tb.N (mm^{-1})	1.38 ± 0.19	1.43 ± 0.24†
Tb.Th (μ)	127.8 ± 15.3	138.4 ± 16.8
Tb.Sp (μ)	609.9 ± 118.7	577.6 ± 125.7
MAR (μm/day)[a]	1.24 ± 0.20	1.34 ± 0.22
MS (%)[a]	22.9 ± 10.4	28.8 ± 9.5
BFR (%/year)[a]	139.1 ± 70.8	185.9 ± 77.7

Note that results from the 6- and 8-week time points have been combined.

[a] Dynamic histomorphometry only available for animals treated with prednisolone for 12 weeks.

* Greater than prednisolone-treated rabbits ($p < 0.10$); † Greater than prednisolone-treated rabbits ($p < 0.05$).

pared with those treated with saline. In addition, the MAR, percent MS, and BFR were 7.7, 25.2, and 20.5% lower, respectively, in the prednisolone group, although these differences did not reach statistical significance (Table 1).

Effects of prednisolone on osteotomy healing

Radiographic, densitometric, biomechanical, and histological comparisons of the untreated ulnas in the prednisolone and saline groups showed that prednisolone treatment dramatically inhibited osteotomy healing. Assessment of the in vivo radiographs showed that none of the untreated ulnas in the prednisolone group achieved radiographic union, whereas 8/23 (35%) of untreated ulnas in the saline group achieved radiographic union ($\chi^2 = 10.8$; $p = 0.001$; Table 2). The area, mineral content, and density of the mineralized callus in the untreated ulnas of rabbits treated with prednisolone were 25, 28, and 3.5% lower, respectively, than corresponding values from untreated ulnas in rabbits treated with saline ($p < 0.001$ for all; Table 3; Fig. 2). Moreover, the torsional strength, stiffness, and energy absorbed to failure were 55–57% lower in the untreated ulnas of the prednisolone-treated rabbits compared with those receiving saline ($p < 0.0001$ for all; Table 3; Fig. 3). During postmortem torsion testing, only 13.6% (3/22) of the untreated ulnas in the prednisolone group fractured outside the original osteotomy in the intact host bone, whereas the remainder of ulnas fractured through the original osteotomy site. In comparison, 55% (11/20) of untreated ulnas in the saline group fractured outside the original osteotomy ($\chi^2=8.1$; $p = 0.005$).

The histological evaluation, although limited to two specimens per group, confirmed the inhibition of healing that was observed radiographically and biomechanically in the prednisolone-treated rabbits. Compared with the untreated ulnas in the saline group, those in the prednisolone group exhibited less extensive bridging of the osteotomy and a less mature callus (Fig. 4). For example, at 6 weeks, the osteotomies in the untreated ulnas of the saline group were bridged primarily with cartilage, whereas those in the prednisolone group were bridged with fibrocartilage. At 8 weeks, the osteotomies in the untreated ulnas of the saline group were bridged with bone and isolated regions of cartilage. In comparison, at this time point, the osteotomies in the prednisolone group were bridged with a combination of cartilage, fibrocartilage, and loose fibrous tissue. The overall callus size, including both the mineralized and the unmineralized portions of the callus, did not appear to be affected by prednisolone treatment. Taken together, these histological observations suggest that the primary effect of prednisolone treatment was to inhibit the overall maturation of the callus and to delay endochondral bone formation.

Effects of rhBMP-2/ACS on osteotomy healing

The effect of rhBMP-2/ACS on osteotomy healing was determined by comparing the ulnas treated with rhBMP-2/ACS with their contralateral untreated ulnas in both the prednisolone and the saline groups. Radiographic, densitometric, biomechanical, and histological results showed that rhBMP-2/ACS enhanced osteotomy healing in both groups, although the effect was larger in the prednisolone group. Poor radiographic healing was observed in the untreated ulnas in both the prednisolone and the saline groups, in which only 16% (8/49) of the limbs achieved radiographic union. In contrast, 80% (39/49) of the rhBMP-2/ACS-treated ulnas achieved radiographic union ($\chi^2=39.3$; $p < 0.0001$ for prednisolone and saline groups combined, Table 2). The number of limbs achieving radiographic union was similar in the rhBMP-2–treated limbs in the prednisolone- and saline-treated groups. Healing was accelerated with rhBMP-2/ACS treatment, because a majority of the ulnas treated with rhBMP-2/ACS showed mineralized callus as early as 2 weeks (81% prednisolone and 91% saline) and showed callus bridging at 3 weeks (58% prednisolone and 65% saline). In comparison, few of the untreated ulnas showed callus formation at 2 weeks (12% prednisolone and 22% saline) and none were bridged with bone at 3 weeks (Table 2).

Properties of the mineralized callus and biomechanical behavior of the healing osteotomy were enhanced by rhBMP-2/ACS treatment in both the saline and the prednisolone groups; however, the magnitude of improvement was greater in the prednisolone group (Table 3). In the prednisolone-treated rabbits, the area, mineral content, and density of the mineralized callus were 40, 43, and 2.7% greater, respectively, in ulnas treated with rhBMP-2/ACS than the contralateral untreated ulnas ($p < 0.0005$ for all; Table 3; Fig. 2). In the prednisolone-treated rabbits, ulnas treated with rhBMP-2/ACS had greater torsional strength (+165%), stiffness (+156%) and energy-to-failure (+199%) compared with the untreated ulnas ($p < 0.0001$ for all; Table 3; Fig. 4). Moreover, 82% (18/22) of limbs treated with rhBMP-2/ACS fractured outside the original osteotomy site compared with only 14% (3/22) of the contralateral untreated limbs ($\chi^2 = 20.5$; $p < 0.0001$).

In comparison, in the saline-treated rabbits, ulnas treated with rhBMP-2/ACS had greater callus area and bone mineral content (+17% for both; $p < 0.02$), but similar callus density as the contralateral untreated ulnas (Table 2, Fig. 2). In the saline group, compared with untreated ulnas, those treated with rhBMP-2/ACS had greater torsional strength

TABLE 2. EVALUATION OF IN VIVO RADIOGRAPHS AT 2, 3, 6, AND 8 WEEKS AFTER OSTEOTOMY

	Saline		*Prednisolone*	
	Untreated	*rhBMP-2/ACS*	*Untreated*	*rhBMP-2/ACS*
Callus Formation at 2 weeks	22% (5/23)	91%‡ (21/23)	12% (3/26)	81%‡ (21/26)
Callus Bridging at 3 weeks	0% (0/23)	65%‡ (15/23)	0% (0/26)	58%‡ (15/26)
Radiographic bridging[a]	52%* (12/23)	100%† (23/23)	12% (3/26)	92%‡ (24/26)
Radiographic union[a]	35%* (8/23)	83%‡ (19/23)	0% (0/26)	77%‡ (20/26)

[a] Refers to whether the osteotomy was identified to be bridged or united at any time.

* Greater than prednisolone untreated ($p < 0.005$ by χ^2 analysis); greater than untreated contralateral † $p < 0.005$ and ‡ $p < 0.0001$ by χ^2 analysis.

TABLE 3. GEOMETRY AND DENSITY OF THE MINERALIZED CALLUS AND BIOMECHANICAL PROPERTIES OF THE HEALING OSTEOTOMY (MEAN ± SD)

	Saline		*Prednisolone*	
	Untreated	*rhBMP-2/ACS*	*Untreated*	*rhBMP-2/ACS*
pQCT evaluation				
Area (mm^2)	14.4 ± 4.2*	16.9 ± 5.3‡	10.8 ± 2.9	15.1 ± 4.4§
Mineral content (mg)	8.32 ± 2.57*	9.76 ± 3.05‡	5.98 ± 1.59	8.56 ± 2.30§
Density (mg/cm^3)	575 ± 18*	577 ± 15	553 ± 9	568 ± 18§
Torsion testing				
Failure torque (N-m)	0.465 ± 0.166†	0.584 ± 0.162‡	0.209 ± 0.156	0.554 ± 0.189§
Torsional stiffness (N-m/deg)	0.021 ± 0.009†	0.024 ± 0.008	0.009 ± 0.008	0.023 ± 0.007§
Energy-to-failure (N-m/deg)	6.24 ± 3.11†	8.38 ± 2.55‡	2.81 ± 2.20	8.41 ± 3.68§

Note that results from the 6- and 8-week time points have been combined.

Greater than prednisolone untreated * $p < 0.005$ and † $p < 0.0005$; greater than untreated contralateral ‡ $p < 0.05$ and § $p < 0.0001$.

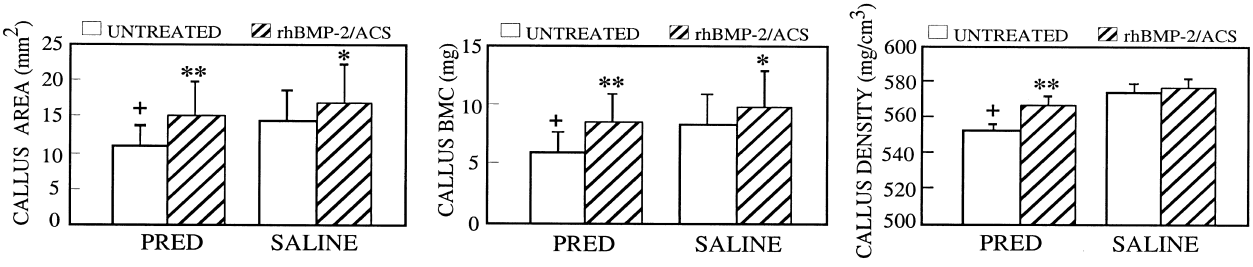

FIG. 2. Area (left), bone mineral content (middle), and density (right) of the mineralized callus in the untreated and rhBMP-2–treated ulnas of prednisolone and saline groups. Error bars represent 1 SD. Results from the 6-week and 8-week cohorts were combined. +prednisolone < saline ($p < 0.001$); *rhBMP-2/ACS > untreated ($p < 0.05$); **rhBMP-2 > untreated ($p < 0.0005$).

(+26%; $p < 0.05$), energy-to-failure (+34%; $p < 0.05$), and torsional stiffness (+14%, NS; Table 2; Fig. 3). Finally, in these saline-treated rabbits, 84% (16/19) of limbs treated with rhBMP-2/ACS fractured outside the original osteotomy site, compared with 55% (11/20) of the contralateral untreated limbs ($\chi^2 = 3.9$; $p < 0.05$).

Histological evaluation confirmed that rhBMP-2/ACS treatment dramatically enhanced healing of the osteotomies in both the prednisolone and the saline groups (Fig. 4). At both the 6-week and the 8-week time points, the ulnas treated with rhBMP-2/ACS were fully bridged with bone. At 6 weeks, the periosteal calluses of the rhBMP-2/ACS treated ulnas were larger than those left untreated. By 8 weeks, the healing process was advanced in the rhBMP-2/ACS-treated ulnas relative to the untreated ulnas, as the periosteal calluses were undergoing remodeling and the endosteal calluses were being resorbed by osteoclasts. Similar remodeling and resorption of the callus was not observed in the untreated osteotomies.

DISCUSSION

In this study we confirmed previous findings that chronic administration of prednisolone inhibits osteotomy healing in rabbits.[10,11] In addition, we showed that a single, local application of rhBMP-2/ACS was able to overcome this inhibition and enhance osteotomy healing. For example, in

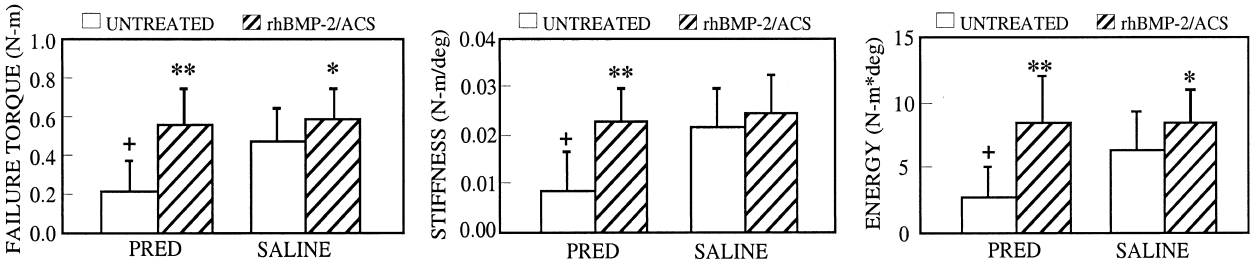

FIG. 3. Failure torque (left), torsional stiffness (middle), and energy absorbed-to-failure (right) of the untreated and rhBMP-2–treated ulnas of prednisolone and saline groups. Error bars represent 1 SD. Results from the 6-week and 8-week cohorts were combined. +Prednisolone < saline ($p < 0.001$); *rhBMP-2/ACS > untreated ($p < 0.05$); **rhBMP-2 > untreated ($p < 0.0001$).

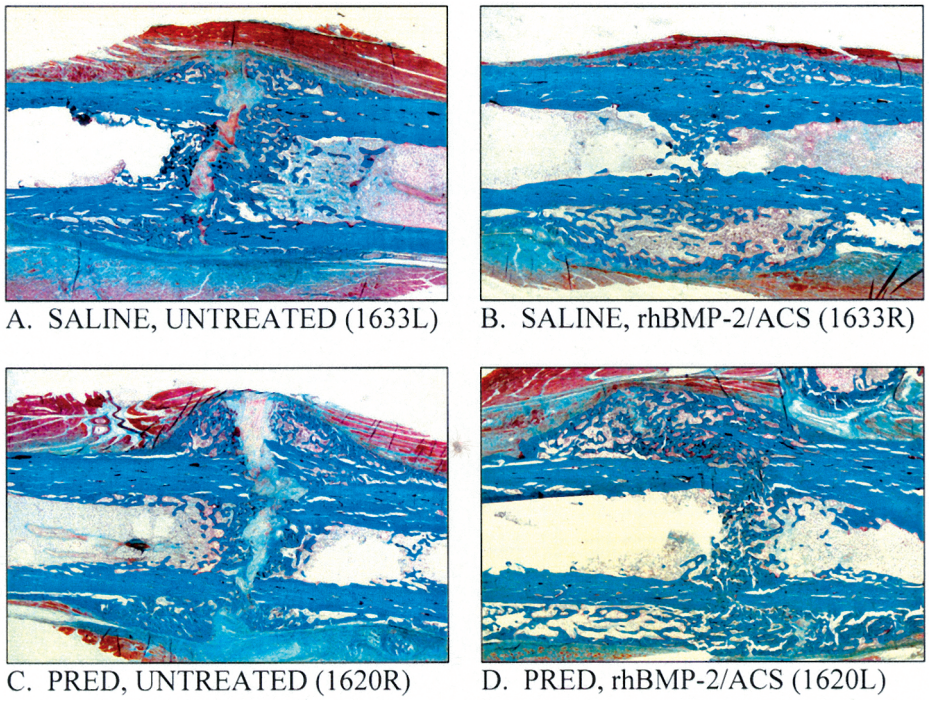

FIG. 4. Representative histological sections of the healing osteotomy in (A and B) saline and (C and D) prednisolone-treated rabbits 6 weeks after creation of the osteotomy (Goldner's trichrome stain, 1×). Note the presence of cartilage in the osteotomy gap in the untreated limbs of the (A) saline and (C) prednisolone rabbit, whereas (B and D) the limbs treated with rhBMP-2/ACS were completely bridged with bone.

the prednisolone-treated rabbits, 65% of the osteotomies treated with rhBMP-2/ACS achieved radiographic union, whereas none of the contralateral untreated osteotomies achieved radiographic union after 6 weeks of healing. Moreover, in rabbits that received prednisolone, the biomechanical properties of osteotomies treated with rhBMP-2/ACS, were at least 2-fold greater than those of the untreated osteotomies. These results show the dramatic positive effect of rhBMP-2/ACS on fracture healing. Although previous studies also have shown the ability of rhBMP-2/ACS to enhance fracture healing,[22,23] to our knowledge, this is the first study that shows the ability of rhBMP-2/ACS to overcome the inhibition of healing associated with glucocorticoid therapy and, furthermore, to enhance healing in this compromised environment. These data have important clinical ramifications because individuals undergoing chronic glucocorticoid therapy are both at increased risk of fracture and increased risk of poor healing of their fractures. Furthermore, these data suggest that rhBMP-2/ACS also may be able to enhance bone healing in other clinical situations in which the healing environment is compromised such as nicotine exposure, alcohol abuse, and diabetes.

Based strictly on a per body weight basis, the dose of prednisolone administered to the rabbits is equivalent to 10 mg/day of prednisone (assuming a body weight of 68 kg or 150 lbs). Ebling et al. reported that rabbits and humans have similar plasma binding of methylprednisolone.[31] Moreover, after adjustment for body weight, the mean residence time and half-life of prednisolone was similar in rabbits and humans. Thus, given the high bioavailability of oral prednisone administration in humans,[32,33] it appears that the prednisolone dose administered to the rabbits in this study is approximately comparable with a 10-mg/day oral prednisone dose in humans. This dose is used commonly for treatment of respiratory, immune, skin, and musculoskeletal disorders.[34]

Our results showing negative effects of glucocorticoid administration on fracture healing concur with previously published findings. In the 1970s, studies in rabbits provided histological evidence of retardation at all stages of fracture

healing after administration of high doses of cortisone (10–25 mg/kg per day).[35,36] More recently, an inhibition of osteotomy healing was observed in rabbits that received prednisolone for 2 months before creation of the osteotomy and also throughout the healing period.[10,11] Our results were similar to those of Waters et al.,[10] who reported that radiographic union was achieved in only 3 of 20 limbs in prednisolone-treated rabbits, whereas 13 of 16 limbs from saline-treated rabbits achieved radiographic union at 6 weeks. Moreover, our observation of a 55–60% decrease in the biomechanical properties of the healing osteotomies in prednisolone-treated rabbits compared with the saline-treated rabbits was nearly identical to the 60–65% decrease previously reported.[10]

In addition to effects on fracture healing, we observed negative effects of prednisolone on bone metabolism in the first months of treatment. Serum osteocalcin levels were 72% lower than baseline levels in the prednisolone-treated rabbits at the time of osteotomy (i.e., after the 6-week pretreatment period). Serum osteocalcin was reduced also (−38%) in the saline-treated rabbits at the time of osteotomy. It is not clear why osteocalcin levels declined in the control rabbits. However, we believe that this gradual decline may be attributable to a stress response associated with the frequent handling for weighing and injections. Nevertheless, the decrease in osteocalcin was greater in the prednisolone-treated rabbits than in the saline-treated rabbits at all time points. In addition to the reduction in serum osteocalcin, we also observed a reduction in vertebral trabecular bone indices. Fourteen weeks after the initiation of prednisolone treatment, there was a significant reduction in vertebral trabecular bone density and trabecular thickness in the prednisolone-treated rabbits. In addition, dynamic indices of bone formation were lower in the rabbits receiving prednisolone, although the differences did not reach statistical significance. This decrease in trabecular bone integrity is consistent with reports of the rapid bone loss seen during the first few months of glucocorticoid treatment in humans.[3,8]

These in vivo studies confirm that glucocorticoid therapy inhibits bone healing. However, the mechanisms by which glucocorticoid therapy inhibits fracture healing are not yet defined. Indications of these mechanisms may be gained from previous studies on the deleterious bone-related effects of glucocorticoids given at supraphysiological concentrations. Both in vitro and in vivo observations suggest that the primary effects of supraphysiological doses of glucocorticoids on bone are to decrease osteoblastogenesis and osteoblast differentiation and enhance apoptosis of existing osteoblasts and osteocytes.[7,37–39] The inhibitory effects of glucocorticoids on fracture healing also may be mediated by suppression of the osteoblast transcription factor Cbfa1,[40] attenuation of type I collagen synthesis and messenger RNA (mRNA) levels,[41–43] decreased synthesis and activity of insulin-like growth factor 1 (IGF-1),[41,44,45] and premature attenuation of the osteoblast cell cycle.[46] Thus, it is likely that glucocorticoids negatively influence fracture healing by affecting various cells involved in the healing process, including osteoblast precursors, preosteoblasts, mature osteoblasts, and osteocytes.

The role of BMP-2 in fracture healing has been described previously.[47,48] It is believed that during normal fracture healing, BMP-2 is released from the bone matrix and periosteum and that it stimulates the differentiation of mesenchymal cells into osteoblasts and chondrocytes. The increased osteoblast differentiation attributed to BMPs has been found to act partly, although not completely, through the up-regulation of Cbfa1.[49] Application of exogenous rhBMP-2 likely enhances fracture healing via the same mechanisms (i.e., increased differentiation of preosteoblasts and up-regulation of Cbfa1) and induction of bone formation. Because expression of Cbfa1 is inhibited after glucocorticoid exposure,[40] either rhBMP-2 overcomes this inhibition or it acts at least partially through pathways that are independent of Cbfa1.[26,50]

Our study cannot explain the molecular mechanisms underlying the effects of glucocorticoids on fracture healing or the potential interaction between glucocorticoids and rhBMP-2. However, it provides an interesting framework for future studies. Among the most important considerations in examining these results are the timing and duration of prednisolone exposure and the profound positive effects of the rhBMP-2/ACS treatment. The prednisolone treatment began 6 weeks before the osteotomy surgery and continued throughout the healing period. In comparison, the rhBMP-2/ACS was applied only once, at the time the osteotomy was created. It should be noted that although the rhBMP-2/ACS was applied at a single time point, Bouxsein and colleagues have shown that small amounts of rhBMP-2 may remain at the osteotomy site up to 2 weeks after implantation.[23] Nonetheless, the prednisolone therapy had several weeks to establish its deleterious effects before BMP-2 was introduced, and therefore rhBMP-2 was acting in an environment with altered bone metabolism, as indicated by the dramatic reduction in osteocalcin levels at the time of osteotomy. Despite this, the rhBMP-2/ACS restored the osteotomies in the prednisolone-treated rabbits to the same degree of enhanced radiographic and biomechanical healing as was seen in the saline-treated rabbits that received rhBMP-2/ACS. Based on this study, we can only suggest possible explanations for this enhancement of fracture healing after rhBMP-2/ACS treatment in rabbits exposed to prednisolone.

One possible explanation for the positive effect of rhBMP-2 in this compromised healing environment may be that the creation of the osteotomy initiated an injury response with recruitment of sufficient mesenchymal cells to overwhelm the glucocorticoid inhibition of osteoblast activity. The differentiation of these recruited mesenchymal cells would have to have been initiated by the pharmacologic doses of rhBMP-2, because endogenous BMP-2 in the limbs of the prednisolone-treated rabbits that did not receive rhBMP-2/ACS was not sufficient to overcome the inhibition associated with prednisolone exposure. Because the prednisolone treatment continued throughout the healing process, previous studies would indicate that bone formation by these newly formed osteoblasts would be inhibited. Thus, either the newly differentiated osteoblasts were resistant to subsequent glucocorticoid exposure or they were present in sufficient number to enhance healing, despite an inhibition in activity. The precise role of rhBMP-2 in overcoming the

glucocorticoid-mediated inhibition of specific osteoblast functions in fracture healing requires additional study.

In summary, we have shown that a single application of rhBMP-2/ACS not only enhances osteotomy healing, but also overcomes inhibition of healing in rabbits exposed chronically to prednisolone. Therefore, rhBMP-2/ACS may prove useful for patients undergoing chronic glucocorticoid therapy, because they are not only at increased risk of fracture, but also are at increased risk of delayed healing or nonunion.

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