Evaluation of serum biochemical markers of bone metabolism for early diagnosis of nonunion and infected nonunion fractures in rabbits

Louise L. Southwood BVSc, PhD; David D. Frisbie DVM, PhD; Chris E. Kawcak, DVM, PhD; C. Wayne McIlwraith BVSc, PhD

Objective—To evaluate the use of serum concentrations of biochemical markers of bone metabolism (osteocalcin [OC], bone-specific alkaline phosphatase [BS-ALP], and deoxypyridinoline [DPYR]) to compare healing in infected versus noninfected fractures and in fractures with normal repair versus delayed (nonunion) repair in rabbits.

Animals—32 female 9- to 10-month-old New Zealand White rabbits.

Procedure—A femoral fracture defect was made in each rabbit. Rabbits were assigned to the following groups: the bone morphogenetic-2 gene treatment group with either noninfected nonunion or infected (ie, inoculation of defects with *Staphylococcus aureus*) nonunion fractures or the luciferase (control) gene treatment group with either noninfected nonunion or infected nonunion fractures. Serum samples were obtained before surgery (time 0) and 4, 8, 12, and 16 weeks after surgery. Callus formation and lysis grades were evaluated radiographically at 16 weeks.

Results—Serum OC and BS-ALP concentrations decreased from time 0 at 4 weeks, peaked at 8 weeks, and then decreased. Serum DPYR concentration peaked at 4 weeks and then decreased, independent of gene treatment group or fracture infection status. Compared with rabbits with noninfected fractures, those with infected fractures had lower serum OC and BS-ALP concentrations at 4 weeks, higher serum OC concentrations at 16 weeks, and higher serum DPYR concentrations at 4, 8, and 16 weeks. Combined serum OC, BS-ALP, and DPYR concentrations provided an accuracy of 96% for prediction of fracture infection status at 4 weeks.

Conclusions and Clinical Relevance—Measurement of multiple serum biochemical markers of bone metabolism could be useful for clinical evaluation of fracture healing and early diagnosis of osteomyelitis. (*J Am Vet Med Assoc* 2003;64:727–735)

Received August 26, 2002.
Accepted December 20, 2002.
From the Orthopaedic Research Laboratory, Department of Clinical Sciences, Colorado State University, Fort Collins, CO 80523. Dr. Southwood's present address is School of Veterinary Medicine, University of Pennsylvania, New Bolton Center, Kennett Square, PA 19348.
Supported by the Morris Animal Foundation.
Presented at the 37th Annual Scientific Meeting of the American College of Veterinary Surgeons, San Diego, October 2002, and the 49th Annual Scientific Meeting of the Orthopaedic Research Society, New Orleans, February 2002.
The authors thank Heather Colhoun, Claire Miller, and Dr. Richard Park for technical assistance.
Address correspondence to Dr. Southwood

Nonunion and infected nonunion fractures are devastating clinical complications following fracture repair in human[1-3] and veterinary[4-7] medicine. In retrospective studies, success rates of < 20% for repair of long-bone fractures in adult horses have been reported.[4-7] Impaired fracture healing (delayed or nonunion fractures) and osteomyelitis (infected nonunion fractures) are the most common causes of failure of treatment of long-bone fractures in horses, with an occurrence rate of 30 to 40% following treatment.[4-7] Approximately 70 to 90% of horses that develop either impaired healing or osteomyelitis are euthanatized.[4-7] Early diagnosis of impaired fracture healing and osteomyelitis is critical for successful treatment.

Although history and physical examination findings may suggest a diagnosis of impaired healing or osteomyelitis, ancillary tests are usually performed to confirm the clinical diagnosis, ascertain whether the infection involves the soft tissue or actual bone, and estimate the prognosis. Radiography is used most commonly; however, radiography is limited by a lack of sensitivity.[8,9] The use of magnetic resonance imaging or computerized tomography has limitations when metallic implants are used to stabilize the fracture, may be cost prohibitive for use in veterinary medicine, and are not commonly available. They also currently require general anesthesia, which is often contraindicated for horses with long-bone fractures because of the risk of damage to the repair during recovery. Nuclear scintigraphy is expensive and requires the use of radioactive material; further evaluation of the accuracy of this imaging modality is required. The use of ultrasonography may result in an accurate diagnosis of osteomyelitis by detection of fluid immediately adjacent to the cortical bone[10,11]; however, ultrasonographic findings lack specificity for detection of postoperative osteomyelitis, because trauma and surgery can result in local fluid accumulation.[10] Therefore, novel methods for early diagnosis of nonunion and infected nonunion fractures are required.

Concentrations of serum and urine biochemical markers of bone metabolism have been evaluated in various diseases of altered bone metabolism,[12-16] including fracture healing.[17-35] An advantage of measuring serum biochemical markers of bone formation and resorption for detection of delayed fracture healing and osteomyelitis is that the results are specific for bone; furthermore, sample collection is easy and economical and does not require general anesthesia or prolonged restraint, compared with some imaging techniques.[17]

However, results from studies evaluating biochemical markers of bone metabolism have been controversial.

Biochemical markers of bone formation include **osteocalcin (OC)** and **bone-specific alkaline phosphatase (BS-ALP)**. Osteocalcin is a specific product of osteoblasts and is associated with bone mineralization.[18,19] A fraction of the OC molecule is released into the blood during the incorporation of OC into bone during bone formation, and serum OC concentration has been shown to increase following fracture.[21-25] In several studies, humans with delayed fracture healing had a lower serum OC concentration after fracture, compared with patients with normal fracture healing, at 0,[20,26] 4,[26] 6,[20] 8,[26] 12,[26] and 16[26] weeks. Also, patients with delayed fracture healing had persistently high serum OC concentrations at 12 weeks, whereas in patients with healed fractures, the serum OC concentration began to decrease.[20]

Bone-specific alkaline phosphatase is an osteoblast enzyme,[23] which is thought to play a role in the formation and mineralization of bone matrix.[30,31] It has been shown to increase after fracture[20,23,24,32,33] and is lower in patients with delayed fracture healing, compared with normal fracture repair, at 4 and 7 weeks[27] and late in the healing process at 20 weeks.[17] In dogs with experimentally infected femoral fractures, serum OC and BS-ALP concentrations are increased, compared with preoperative values.[29]

Biochemical markers of bone resorption include **deoxypyridinoline (DPYR)** crosslinks, which are specific for bone and dentine.[21] During bone resorption, collagen is degraded, and DPYR is released into the blood. An increase in serum and urine DPYR concentration has been reported after fracture,[21,25] with peak concentrations reported at 2 to 6 weeks[25] and 4 to 8 weeks[21] after fracture. To our knowledge, urine and serum concentrations of DPYR have not been evaluated in patients with osteomyelitis.

Concentrations of other biochemical markers of bone formation, such as total alkaline phosphatase, procollagen type-I carboxy-terminal peptide, and type-III amino-terminal peptides, change following fracture but are not specific for bone.[36] Similarly, measurement of tartrate-resistant acid phosphatase, pyridinoline crosslinks, hydroxyproline, hydroxylysine, and urine calcium concentrations, which are biochemical markers of bone resorption, has several limitations including lack of specificity for bone, complex metabolism, technical difficulties in concentration measurement, and the requirement of a urine sample, which can be more difficult to obtain than a venous blood sample in large animals.[36] The serum concentration of carboxy- and amino-terminal type-I collagen telopeptides increases following fracture and with the development of osteomyelitis; however, there is the limitation of antibody cross-reactivity for some species when using the commercially available kits.[36]

To our knowledge, no studies have been published in which serum bone marker concentrations were evaluated in animals with nonunion or infected nonunion fractures. The purpose of the study reported here was to evaluate the use of serum concentrations of biochemical markers of bone metabolism to compare healing in infected versus noninfected fractures and in fractures with normal repair versus delayed (nonunion) repair in rabbits. Our hypotheses were that serum biochemical markers of bone formation (OC and BS-ALP) and resorption (DPYR) would be significantly different in rabbits with nonunion fractures, compared with those with normal fracture repair, and in rabbits with infected fractures, compared with those with noninfected fractures.

Materials and Methods

Animals—This study was performed as part of a larger study to evaluate the effect of **adenoviral transfer (Ad)** of the **bone morphogenetic-2 (*BMP*-2)** gene[a] on fracture healing in rabbits with noninfected nonunion and infected nonunion fractures.[b] Direct in vivo gene transfer was used, and transduction of cells at the fracture defect site was previously evaluated in a similar study.[37] All procedures were approved by the institutional animal care and use committee.

Thirty-two skeletally mature female (9- to 10-month old) New Zealand White rabbits were used in the study. Rabbits were assigned to the following treatment groups: the **Ad-*BMP*-2 (BMP)** gene treatment group (ie, BMP-group rabbits) with either noninfected nonunion or infected nonunion fractures or the **Ad-luciferase (LUC)** control gene treatment group (ie, LUC-group rabbits) with either noninfected nonunion or infected nonunion fractures.

Rabbits were premedicated with morphine (0.5 mg/kg, SC) and glycopyrrolate (0.03 mg/kg, SC), and anesthesia was induced and maintained by use of isoflurane in oxygen. A routine lateral surgical approach to the left femur was used. A 10-mm-long mid-diaphyseal femoral defect was surgically created by use of a side-cutting carbide burr.[c] The periosteum was removed by use of a periosteal elevator, and the endosteum and bone marrow were removed by use of curettage. The femur was stabilized by stacking two 2.0-mm cuttable bone plates and using eight 2.0-mm-diameter cortical screws, with cerclage wire placed proximally and distally.[37,b] A sclerosing agent (sodium morrhuate) was used on the end of the proximal and distal fragments in all rabbits to prevent defect ossification in LUC-group rabbits and facilitate development and persistence of infection in rabbits with infected fractures.

Perioperative analgesia consisted of preoperative administration of morphine epidurally (0.1 mg/kg), intraoperative administration of fentanyl as an IV bolus (0.02 mg/kg), followed by a constant rate infusion (20 µg/kg/h), and postoperative administration of flunixin meglumine (0.5 mg/kg, SC) for 72 hours or as needed. Butorphanol tartrate (0.4 mg/kg, SC) was also administered as needed after surgery. A single dose of enrofloxacin (10 mg/kg, SC) was administered before surgery to reduce the risk of infection from surgical contaminants. A scoring system was used to assess signs of systemic illness and lameness after surgery.

At 48 hours after surgery, femoral defects were percutaneously inoculated via palpation with 0.5×10^7 colony-forming units of *Staphylococcus aureus* (S. Schaefler 1428, ATCC #25923)/0.5 mL.[b] Gene treatment of fracture defects with either Ad-luciferase or Ad-*BMP*-2 was also administered percutaneously at the time of inoculation with *S aureus*. Rabbits were euthanatized with pentobarbital (88 mg/kg, IV) 16 weeks after surgery. At necropsy, the fracture was evaluated for gross signs of infection. Tissue surrounding the fracture site underwent **quantitative aerobic culture (QAC)**; a QAC > 10^4 colony-forming units/mL was used to define infection.

Body weight was recorded before surgery and at the time of euthanasia. Rabbits were graded for lameness (0 = none, 1 = slight, 2 = mild, 3 = moderate, 4 = severe or nonweight-bearing) at 4, 8, 12, and 16 weeks after surgery.

Blood sample collection—Blood samples were collected before surgery (time 0) and at 4, 8, 12, and 16 weeks after surgery. Blood samples were collected in the morning during a 1- to 2-hour period. Blood was collected into 12-mL volume syringes via a 20-gauge catheter placed in the auricular artery and then immediately transferred to a serum collection tube, placed on ice, and refrigerated (4°C). Following clot formation, samples were centrifuged at 4°C, and serum aliquots were stored at –80°C. All serum samples were analyzed at the completion of the study.

Serum samples were obtained from blood collected at 16 weeks from an additional 24 rabbits that were part of a concomitant study, which included the same surgical techniques, *S aureus* inoculation methods, BMP- and LUC-gene treatment groups, and postoperative management and evaluation as those described here.[b] The additional 22 rabbits were grouped as follows: 6 BMP-group rabbits with noninfected fractures, 7 LUC-group rabbits with noninfected fractures, 7 BMP-group rabbits with infected fractures, and 2 LUC-group rabbits with infected fractures. Serum samples from rabbits of the concomitant study were analyzed, and the results were included in the 16-week analysis to increase statistical power.

Measurement of serum biochemical markers of bone metabolism—Concentrations of serum biochemical markers of bone metabolism were measured by use of various ELISAs. The OC assay[d] was a competitive immunoassay that included OC-coated microtiter strips, a mouse anti-OC antibody, and an anti-mouse IgG-alkaline phosphatase conjugate. p-nitrophenyl phosphate was used as a substrate for alkaline phosphatase, and the reaction resulted in a yellow color with the intensity inversely related to the concentration of OC in serum samples. The **optical density (OD)** of standards, serum samples, and controls was measured at 405 nm[e], and a 4-parameter calibration curve was plotted from the standards.[f] The concentration of OC (ng/mL) in serum samples and controls was calculated from a 4-parameter calibration curve. The BS-ALP assay[g] included a monoclonal antibody coated on the microtiter strip. The monoclonal antibody bound the BS-ALP in serum samples. p-Nitrophenyl phosphate was used as a substrate for bound BS-ALP. The OD of standards, serum samples, and controls was measured at 405 nm. A standard curve was generated from standards, and the concentration (U/L) of BS-ALP in controls and serum samples was calculated from the standard curve.[e] Total serum DPYR concentration was measured by use of a competitive ELISA.[h] The DPYR in serum samples competed with alkaline phosphatase-conjugated DPYR for binding to monoclonal anti-DPYR antibodies coated on the strip. The reaction was detected with p-nitrophenyl phosphate substrate, and the concentration of DPYR was calculated from a 4-parameter standard curve[e] following OD measurement of standards, serum samples, and controls at 405 nm. Serum samples were analyzed in duplicate.

Radiographic evaluation—Fractures were evaluated radiographically (craniocaudal and lateral views) 16 weeks after surgery.[b] Radiographs were taken with the rabbits under general anesthesia by use of isoflurane in oxygen. Radiographs were evaluated by a board certified veterinary radiologist (Dr. Richard Park) who was unaware of the experimental group assignment. Radiographic grades (0 = none, 1 = slight, 2 = mild, 3 = moderate, 4 = severe) for bone lysis and external callus formation were evaluated at the completion of the study (16 weeks). Our objective was to evaluate the use of serum biochemical markers of bone metabolism for predicting osteomyelitis and the degree of fracture healing at the end of the study (ie, at 16 weeks); none to minimum bone lysis was observed early in the study, and bone lysis and external callus grade increased throughout the study, reaching a maximum at 16 weeks. Bone lysis was considered as none (grade 0) if there was no lysis; slight (grade 1) if it was associated with the bone adjacent to a single screw or confined to the bone immediately adjacent to the defect; mild (grade 2) if bone lysis was associated with the bone adjacent to multiple screws, the defect, or the bone plate; moderate (grade 3) if bone lysis was associated with the bone in between the screws but was not extensive; and severe (grade 4) if bone lysis was associated with the entire bone. External callus formation was considered as none (grade 0) if there was no callus; slight (grade 1) if < 2 cm of thin callus formed on the proximal fragment, distal fragment, or both; mild (grade 2) if > 2 cm of thin callus extended from the proximal and distal fragment; moderate (grade 3) if > 2 cm of thick callus extended from the proximal and distal fragment; and marked (grade 4) if a thick bridging callus was observed. External callus was also classified as bridging or not bridging, and a bridging callus was defined as one in which a callus was uniting the proximal and distal fragments

Statistical analysis—Data were analyzed by use of an ANOVA.[i] Rabbit, time (0, 4, 8, 12, 16 weeks), gene treatment group (BMP- or LUC-group rabbits), and fracture infection status (infected or noninfected) were used as class variables. Nesting of data from rabbits within gene treatment group and infection status was used as the random variable. Data were analyzed by use of several models to evaluate the association between marker concentration (dependent variable) and gene treatment group, time, and interactions; between marker concentration and radiographic lysis grade (0 to 4), time, and interactions; between marker concentration and radiographic callus grade (0 to 4), time, and interactions; and between marker concentration and bridging callus formation, time, and interactions (fixed effects). An overall analysis as well as an analysis at each blood sample collection time was performed.

Data were analyzed for normalcy by use of a plot of the predicted versus residual values; if the data did not appear normally distributed, a transformation was performed and data were reanalyzed. If an increase in variance with an increase in serum concentration was found, a log transformation of the data was performed. The difference in log value between time 0 and each subsequent blood sample collection time was calculated and represented the change in serum bone marker concentration. The association between fixed effects and the change in serum bone marker concentration was analyzed. The association between preoperative body weight, body weight at the time of euthanasia, change in body weight, and lameness grade were also evaluated with the developed models by use of an ANCOVA. If a significant association between serum bone marker concentration and body weight or lameness grade was found, these data were included in the analysis.

Correlations between different marker concentrations and external callus grade, lysis grade, body weight, and lameness grade were analyzed by use of the Pearson correlation coefficient.[j] The probability of serum OC, BS-ALP, and DPYR concentrations providing a prediction as to whether a rabbit had an infected fracture or a bridging callus at 16 weeks was determined by use of logistic regression,[k] and the accuracy, true-positive (sensitivity), true-negative (specificity), and positive and negative predictive values were calculated.[l] Data are presented as least square mean values. A value of $P < 0.05$ was considered significant.

Results

Animals and fracture healing—Of the 32 rabbits, 20 completed the 16-week study as follows: 5 BMP-group rabbits with noninfected fractures, 7 LUC-group

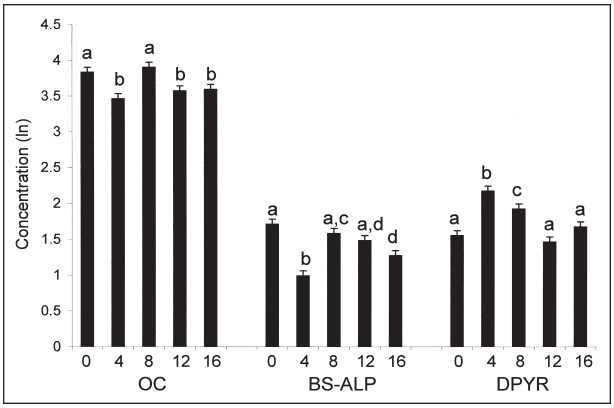

Figure 1—Association between log (ln) values of least square mean (LSM [± SEM]) serum concentrations of osteocalcin (OC), bone-specific alkaline phosphatase (BS-ALP), and deoxypyridinoline (DPYR) and time averaged over gene treatment group and infection status. Blood samples from rabbits were obtained before surgery (time 0) and at 4, 8, 12, and 16 weeks after surgery. [a,b,c,d]Different letters represent significantly ($P < 0.05$) different serum concentrations for each bone marker.

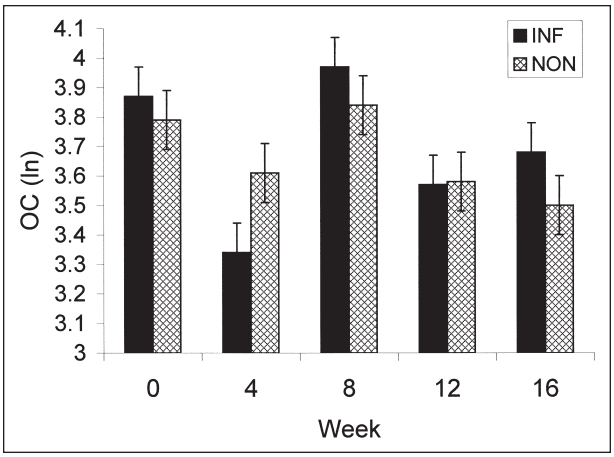

Figure 2—Association between infection status (infected [INF] and noninfected [NON] fractures) in rabbits and log (ln) values of LSM (± SEM) serum OC concentrations at time 0 and 4, 8, 12, and 16 weeks after surgery.

rabbits with noninfected fractures, 3 BMP-group rabbits with infected fractures, and 5 LUC-group rabbits with infected fractures. Twelve of the 32 rabbits were euthanized for humane reasons prior to study completion.[b] Four of the 12 rabbits were euthanized after week 4; therefore, data from these rabbits were included until the time of euthanasia.

Fractures healed predominantly by external callus formation rather than by defect healing. Therefore, callus grade at 16 weeks and the formation of a bridging callus were used as a measure of fracture healing.

Osteocalcin—A significant ($P < 0.001$) association was found between time and serum OC concentration, independent of gene treatment group or fracture infection status (**Fig 1**). Independent of gene treatment group, rabbits with infected fractures had a lower serum OC concentration at week 4 and higher serum OC concentration at week 16, compared with rabbits with noninfected fractures, but this difference was not significant (**Fig 2**). When the change in serum OC concentration from time 0 was evaluated, a significant ($P = 0.02$) association was found with infection status at week 4; rabbits with infected fractures had a greater decrease in serum OC concentration, compared with rabbits with noninfected fractures. A significant ($P = 0.01$) association was found between serum OC concentration and preoperative body weight; when preoperative body weight was included in the analysis, the interaction between fracture infection status and time was significant ($P = 0.05$), and at 4 weeks, a significant ($P = 0.04$) association was found between fracture infection status and serum OC concentration, as well as between fracture infection status and change in serum OC concentration from time 0 ($P = 0.02$). Overall, serum OC concentration was not useful for predicting fracture infection status, compared with serum BS-ALP and DPYR concentrations (**Table 1**). No association was found between gene treatment group and serum OC concentration.

No association or correlation was found between lysis grade and serum OC concentration. Rabbits with a bridging callus did not have a significantly different serum OC concentration, compared with rabbits that did not have bridging callus. At 16 weeks, rabbits with a bridging callus had a lower serum OC concentration, compared with rabbits that did not have a bridging callus, but this difference was not significant ($P = 0.08$). The serum OC concentration was not useful for differentiating rabbits with and without a bridging callus. No association or correlation was found between serum OC concentration and external callus grade.

Bone-specific alkaline phosphatase—A significant ($P < 0.001$) association was found between time and serum BS-ALP concentration, independent of gene treatment group or fracture infection status (Fig 1). A significant ($P = 0.04$) association was found between fracture infection status and serum BS-ALP concentration, as well as a significant ($P < 0.001$) interaction between fracture infection status and time; rabbits with infected fractures had significantly lower serum BS-ALP concentrations at 4 weeks, compared with rabbits with noninfected fractures (**Fig 3**). The change in serum BS-ALP concentration from time 0 was also significantly ($P < 0.001$) greater for rabbits with infected fractures at 4 weeks. When body weight at euthanasia or change in body weight from time 0 was included in the model, rabbits with infected fractures still had a lower serum BS-ALP concentration at 4 weeks, compared with rabbits with noninfected fractures. A moderate correlation ($P < 0.001$; $r^2 = 0.47$) was found between serum BS-ALP concentration at 4 weeks and body weight at euthanasia, as well as between serum BS-ALP concentration and change in body weight from time 0 ($P < 0.001$; $r^2 = 0.5$). Serum BS-ALP concentration could be used to differentiate rabbits with infected fractures from those with noninfected fractures at 4 weeks, but not at other times (Table 1). No association was found between serum BS-ALP concentration and gene treatment group.

A significant ($P = 0.005$) association between lysis grade and serum BS-ALP concentration, as well as between lysis grade and change in serum BS-ALP con-

Table 1—Accuracy, sensitivity, specificity, and positive and negative predictive values for serum osteocalcin (OC), bone-specific alkaline phosphatase (BS-ALP), and deoxypyridinoline (DPYR) concentrations for predicting fracture infection status in rabbits

Serum bone marker	Time (wks)	Values				
		Accuracy (%)	Sensitivity (%)	Specificity (%)	Positive predictive (%)	Negative predictive (%)
OC	4†	67	45	85	71	65
OC	8	48	33	58	38	54
OC	12	60	0	100	0	60
OC	16†	66	33	88	67	66
BS-ALP	4*	75	73	77	73	77
BS-ALP	8	62	11	100	100	60
BS-ALP	12	60	0	100	0	60
BS-ALP	16	59	0	100	0	60
DPYR	4*	75	64	85	78	73
DPYR	8 *	81	78	83	78	83
DPYR	12†	70	50	83	67	71
DPYR	16*	75	61	85	73	76
Combined	4*	96	91	100	100	93
Combined	8†	71	67	75	67	75
Combined	12*	75	50	92	80	73
Combined	16*	75	61	85	73	76

*Significantly ($P < 0.05$) different concentration of serum biochemical markers of bone metabolism between rabbits with infected fractures and rabbits with noninfected fractures at each measurement time.
†$P < 0.10$.

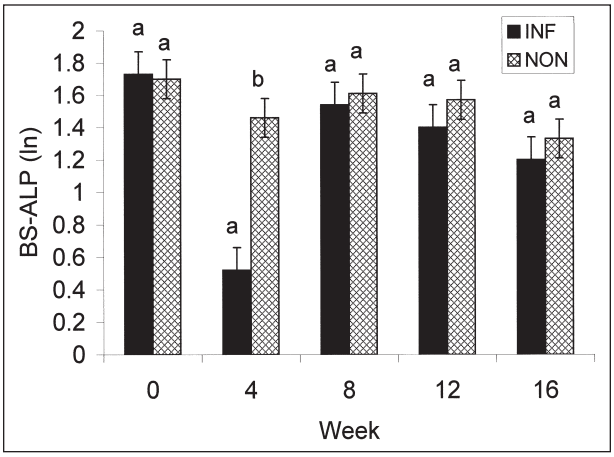

Figure 3—Association between INF and NON fractures in rabbits and log (ln) values of LSM (± SEM) serum BS-ALP concentrations at time 0 and 4, 8, 12, and 16 weeks after surgery. [a,b]Different letters represent significantly ($P < 0.05$) different serum concentrations of BS-ALP at a measurement time between rabbits with infected fractures and rabbits with noninfected fractures.

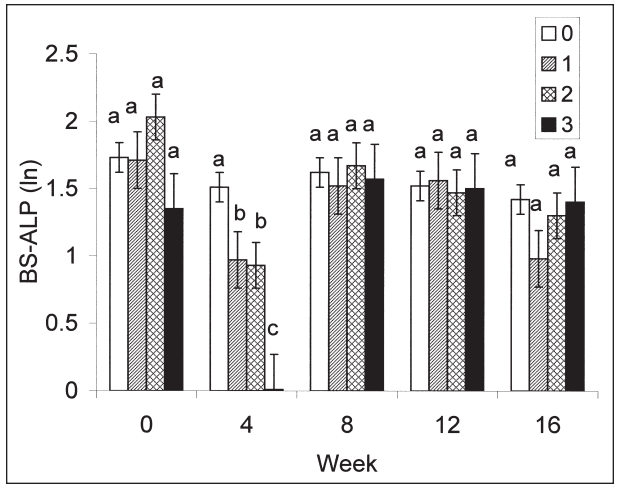

Figure 4—Association between radiographically determined fracture lysis grades (0 = none, 1 = slight, 2 = mild, 3 = moderate, 4 = severe) of rabbits and log (ln) values of LSM (± SEM) serum BS-ALP concentrations at time 0 and 4, 8, 12, and 16 weeks after surgery. [a,b,c]Different letters represent significantly ($P < 0.05$) different serum concentrations of BS-ALP at a measurement time among rabbits with various lysis grades.

centration from time 0, was found at 4 weeks ($P = 0.007$); rabbits with a higher lysis grade had a lower serum BS-ALP concentration **(Fig 4)**. At 4 weeks, a moderate negative correlation ($r^2 = -0.45$; $P = 0.001$) was found between lysis grade and serum BS-ALP concentration, as well as between lysis grade and change in serum BS-ALP concentration from time 0 ($r^2 = -0.48$; $P < 0.001$).

No difference in serum BS-ALP concentration was found between rabbits that had bridged the defect at 16 weeks and those that had not bridged the defect; serum BS-ALP concentration was not useful for predicting which rabbits had and which did not have a bridging callus by use of logistic regression. A significant ($P = 0.05$) association was found between the 16-week radiographic external callus grade and serum BS-ALP concentration; rabbits with grade-4 callus formation had a higher serum BS-ALP concentration, compared with rabbits with a lower callus grade.

DPYR—A significant ($P < 0.001$) association was found between time and serum DPYR concentration, independent of gene treatment group or fracture infection status (Fig 1). Rabbits with infected fractures had a significantly ($P < 0.001$) higher serum DPYR concentration at weeks 4, 8, and 16, compared with rabbits with noninfected fractures **(Fig 5)**. The serum DPYR concentration was the most useful serum bone marker for differentiating rabbits with infected fractures from those with noninfected fractures (Table 1).

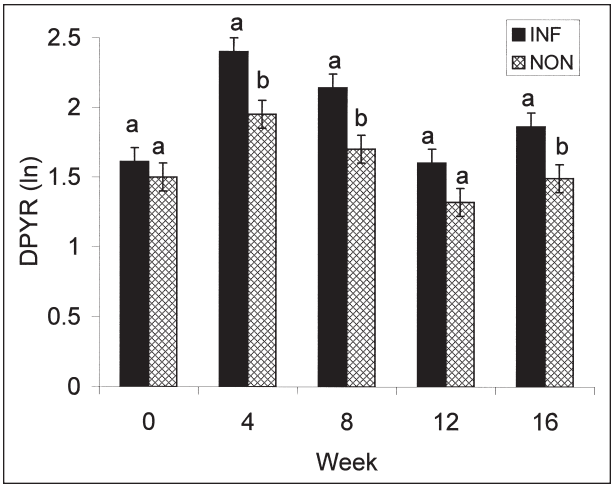

Figure 5—Association between infection status INF and NON fractures in rabbits and log (ln) values of LSM (± SEM) serum DPYR concentrations at time 0 and 4, 8, 12, and 16 weeks after surgery. [a,b]Different letters represent significantly ($P < 0.05$) different serum concentrations of DPYR at a measurement time between rabbits with infected fractures and rabbits with noninfected fractures.

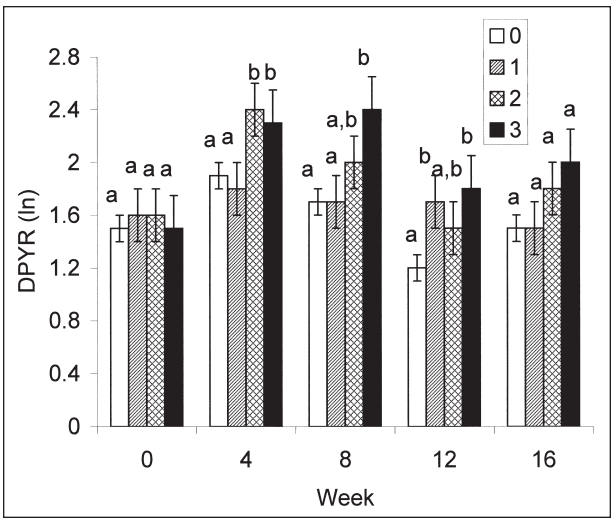

Figure 6—Association between radiographically determined fracture lysis grades (0 = none, 1 = slight, 2 = mild, 3 = moderate, 4 = severe) of rabbits and log (ln) values of LSM (± SEM) serum DPYR concentrations at time 0 and 4, 8, 12, and 16 weeks after surgery. [a,b]Different letters represent significantly ($P < 0.05$) different serum concentrations of DPYR at a measurement time among rabbits with various lysis grades.

Although no association was found between gene treatment group and serum DPYR concentration, a significant ($P = 0.02$) 3-way interaction was found between fracture infection status, gene treatment group, and time. Rabbits with infected fractures had an increase in serum DPYR concentration, which remained increased, compared with rabbits with noninfected fractures; BMP-group rabbits also had an increase in serum DPYR concentration, which then decreased after week 4.

A significant ($P = 0.001$) association was found between radiographic lysis grade and serum DPYR concentration (**Fig 6**); rabbits with a higher lysis grade had a higher serum DPYR concentration. A moderate correlation ($r^2 = 0.33$; $P = 0.008$) was found between lysis grade and serum DPYR concentration at week 4, and only a weak correlation was found thereafter ($r^2 = 0.24$).

No difference was found in serum DPYR concentration in rabbits with a bridging callus, compared with rabbits without a bridging callus. The serum DPYR concentration was not useful for differentiating rabbits with a bridging callus from those without by use of logistic regression. Rabbits with a higher callus grade had a higher serum DPYR concentration at 4 weeks, and rabbits with a bridging callus (grade 4) had an earlier decrease in serum DPYR concentration; however, these findings were not significant. No correlation was found between serum DPYR concentration and callus grade at any time.

A weak positive correlation was found between serum OC and BS-ALP concentrations at 4 weeks ($r^2 = 0.23$, $P = 0.02$), and a moderate positive correlation ($r^2 = 0.42$; $P < 0.001$) was found between the change in serum OC and BS-ALP concentrations from time 0 at 4 weeks. No correlation was found between serum OC or BS-ALP concentrations and serum DPYR concentration.

Combination of serum biochemical markers of bone metabolism—The accuracy of a combination of serum OC, BS-ALP, and DPYR concentrations for predicting fracture infection status was determined. When a combination of serum biochemical markers of bone metabolism was used to differentiate rabbits with infected fractures from those with noninfected fractures at 4 weeks, the accuracy was 96% (Table 1).

Discussion

Results of our study indicate that serum biochemical markers of bone metabolism may be useful for evaluating the development of postoperative osteomyelitis. Rabbits with infected fractures had lower serum OC and BS-ALP concentrations and a higher serum DPYR concentration at 4 weeks, compared with rabbits with noninfected fractures. When a combination of serum biochemical markers of bone metabolism was used to predict whether rabbits had infected fractures, the overall accuracy was excellent at 4 weeks and higher than that previously reported with imaging modalities at this time.[8-10] The benefit of the use of serum biochemical markers of bone metabolism for detecting early osteomyelitis was supported by the lack of radiographic evidence of bone lysis in rabbits with fractures that were infected early in the healing process, emphasizing the lack of sensitivity of radiography.[b] The low serum OC and BS-ALP concentrations reflect a reduction in bone formation in rabbits with infected fractures. These findings are supported by nuclear scintigraphy findings of another study[6] in which a decrease in the uptake of technetium labeled diphosphonate, a measure of bone formation, at 4 weeks correlated with an increase in radiographic lysis grade at 16 weeks.

In our study, the low serum BS-ALP concentration at 4 weeks may be an indication of the negative effect of systemic disease on bone formation. Changes in serum OC concentration followed a similar pattern to changes in serum BS-ALP concentration at 4 weeks.

However, serum OC concentrations were high in rabbits with infected fractures at 16 weeks, whereas serum BS-ALP concentrations were not. The increase in serum OC concentration in rabbits with infected fractures at 16 weeks may be a combination of OC release as a result of bone resorption, as well as bone formation associated with sclerosis and proliferation. Measurement of serum OC concentration was found to be of no benefit in evaluating chronic osteomyelitis in humans because of the wide ranges in concentration and various factors affecting serum OC concentration.[28] In dogs with an infected femoral fracture that was stabilized with an intramedullary pin, serum OC and BS-ALP concentrations were increased 1 and 2 weeks after fracture and infection initiation, reaching a peak at 2 weeks.[29] However, the duration of the study was only 4 weeks, and no dogs with noninfected fractures were included. Therefore, it was difficult to evaluate the effect of fracture versus fracture infection on serum bone marker concentrations.

In our study, serum DPYR concentrations were higher in rabbits with infected fractures at all times, compared with those with noninfected fractures, which is a reflection of bone degradation; rabbits with a high radiographic lysis grade had a significantly higher serum concentration of DPYR. The difference in serum DPYR concentrations between rabbits with infected fractures and those with noninfected fractures was not significant at 12 weeks, and the reasons for this are unknown; however, it may have been a physiologic phenomenon associated with several factors that can affect serum bone marker concentration or a type-II statistical error. Our finding is similar to results of another study in which an increase in serum concentrations of type-I collagen telopeptides were associated with infected fractures.[29] The type-I collagen telopeptides are small peptide fragments on the end of the collagen fibril and are also released into the blood during bone resorption. Serum concentrations of type-I collagen telopeptides were not evaluated in our study, because there are no commercially available kits to measure serum concentrations of type-I collagen telopeptides in rabbits; however, type-I collagen telopeptides, when used in combination with other serum biochemical markers of bone metabolism, could further improve the accuracy of detecting postoperative osteomyelitis in clinically affected animals.

Measurements of serum concentrations of biochemical markers of bone metabolism may also be useful for evaluating fracture healing, although the results in our study were not as favorable as expected. Rabbits with high callus grades generally had high serum BS-ALP concentrations. Although a high serum OC concentration at 8 and 12 weeks and a high serum DPYR concentration at 4 and 8 weeks was found in rabbits that had a high external callus grade, this finding was not significant in our study because of the large variability in data. Rabbits with a grade-4 callus had the highest concentrations of serum biochemical markers of bone metabolism, and then after reaching a peak, a decrease in these values was observed, which may have represented formation of a bridging callus. Rabbits with a bridging callus had a decrease in serum OC concentrations from time 0 at 16 weeks. These results support those of another study[20] in which the decrease in serum bone marker concentration was more rapid in patients with normal fracture repair, compared with patients with delayed (nonunion) fracture repair. In rabbits with infected fractures in our study, the increase in serum DPYR concentration associated with an increase in callus formation may have been a reflection of the interaction between bone lysis and bone proliferation and callus formation. Alternatively, a large callus may provide more bone for resorption and, consequently, a high serum concentration of DPYR. Results of another study[34] indicate that serum type-I collagen telopeptide concentrations increase after fracture and patients with delayed fracture healing have higher serum concentrations at 2 weeks after fracture. Therefore, measurements of serum bone marker concentrations over time may be more useful than the concentration at a given point.

In our study, a significant change was found in all serum concentrations of biochemical markers of bone metabolism over time. Serum biochemical markers of bone formation (BS-ALP and OC) decreased at week 4, peaked at week 8, and then decreased to values lower than those at time 0, whereas the marker of bone resorption (DPYR) peaked at week 4 and then decreased. These results indicate that in general, an initial phase of bone resorption with minimal bone mineralization occurred, particularly in rabbits with infected fractures, followed by bone formation, reflecting the phases of fracture healing.[21] These results are supported by those of previous studies. For instance, in studies[23,32] evaluating humans with normally healing tibial shaft fractures, serum BS-ALP concentration initially decreased at 1 week and then increased throughout the study, and serum OC concentration increased after fracture and then decreased, reaching a minimum concentration at week 5, and then increased thereafter. In another study,[20] serum OC and ALP concentrations were higher at 6 weeks, compared with immediately after fracture, and at 12 weeks, serum concentrations were not different from concentrations before surgery. Urine DPYR concentration was found to increase 1 week after fracture, peak between 4 and 8 weeks, and return to reference range values by week 24.[21]

The initial high serum BS-ALP and OC concentrations at time 0, compared with concentrations at week 16, are difficult to interpret; however, it probably reflects the young age of the rabbits. The rabbits used in our study were skeletally mature (ie, 9 months old), and the femoral growth plates were closed, but these rabbits may still have been actively mineralizing bone. It is also possible that the higher serum OC and BS-ALP concentrations may have been related to activity during transportation or changes in housing.

The physiologic processes associated with the release of biochemical markers of bone metabolism into the blood are complex, and there are numerous factors that affect serum bone marker concentration. Changes in concentrations of serum biochemical markers of bone metabolism after fracture reflect bone resorption and formation associated with the actual

fracture and repair, osteonecrosis associated with the initial injury and surgery, lameness, reduced activity, immobilization or physical unloading of the limb, bone remodeling, fracture infection status, hemorrhage (including sample blood collection), and systemic disease.[21] Interestingly, no effect of lameness on serum concentration of bone markers was found in our study; however, the effect of reduced weight-bearing on the affected limb may have been counteracted by altered gait and an increase in weight-bearing on the contralateral limb. Further, the lack of association between serum bone marker concentration and callus and lysis grades may also be a result of interactions between bone formation and resorption with healing and osteomyelitis.

Circadian, seasonal, age-related, and hormonal factors, as well as various methods of blood sample collection and analysis, also affect bone marker concentration. A large variability between individuals in serum bone marker concentration following fracture and during fracture repair has been reported[34,35] and was also found in our study. The large variability in data between individuals as well as interassay variability may have resulted in the low number of significance differences in values among groups of rabbits in our study.

The application of information from our study in rabbits to other clinically affected animals, with respect to changes in serum bone marker concentration, is unknown. An osteoinductive treatment was used in our study, and to our knowledge, no studies have been conducted to evaluate the association between gene treatment with Ad-*BMP*-2 and serum bone marker concentration. Although results of a previous study[37] indicate that there is a high and consistent transduction of cells in the fracture defect region when the *BMP*-2 gene is delivered by use of an adenoviral vector, this was not evaluated in our study. However, we used external callus and lysis grades to determine the association between serum bone marker concentrations and fracture healing and infection status; therefore, if variability in cell transduction and Ad-*BMP*-2 gene production occurred, the association with gene treatment group would have been affected, but not the association with measurements of fracture healing and infection status. Interestingly, although no association was found between serum DPYR concentration and gene treatment group, a 3-way interaction was found between serum DPYR concentration and fracture infection status, gene treatment group, and time. This may have been a type-I statistical error or the *BMP*-2 gene may have actually caused an increase in serum DPYR concentration by increasing bone resorption through enhancing blood supply or osteoclastic activity. Rabbits used in our study had severe nonunion fractures, a large defect, and damage to the bone and vascularity.[b] Rabbits healed by a bridging external callus rather than by defect ossification.[b] Different types of fractures result in differing changes in serum bone marker concentrations. For example, vertebral compression fractures in humans do not alter serum OC concentration[21]; hip fracture repair in humans results in an increase in serum OC concentration, whereas hip prosthesis does not, possibly because the fracture site is removed.[35] Further, the high accuracy of serum concentration measurements at 4 weeks in our study was not reflected at later times. Considerable variability in serum concentrations of bone markers would be found in clinically affected animals, the effect of which could not be determined in our study.

[a]The adenoviral vector with the *BMP*-2 gene was provided by Dr. Chris Evan's Laboratory, Harvard University, Boston, Mass.
[b]Southwood LL. Gene therapy for treatment of infected non-unions and novel methods for early diagnosis of impaired fracture healing PhD dissertation, Colorado State University, Fort Collins, Colo, 2002.
[c]MicroAire Surgical Instruments, Charlottesville, Va.
[d]Osteocalcin, Quidel Corp, Santa Clara, Calif.
[e]Dynex Revelation 3.2, Dynex Technologies Inc, Chantilly, Va.
[f]Metra Fit 1.1, Metra Biosystems Inc, Mountain View, Calif.
[g]Alkphase-B, Quidel Corp, Santa Clara, Calif.
[h]Total DPD and DPD, Quidel Corp, Santa Clara, Calif.
[i]PROC MIXED, SAS Institute, Cary, NC.
[j]PROC CORR, SAS Institute, Cary, NC.
[k]PROC PROBIT, SAS Institute, Cary, NC.
[l]PROC FREQ, SAS Institute, Cary, NC.

References

1. Yelin E, Callahan LF. The economic cost and social and psychological impact of musculoskeletal conditions. *Arthritis Rheum* 1995;38:1351–1362.
2. Praemer A, Furner S, Rice DP. *Musculoskeletal conditions in the United States*. Park Ridge, Ill: American Academy of Orthopaedic Surgeons, 1992;85–124.
3. Johnson EE, Urist MR, Finerman AM. Resistant nonunion and partial or complete segmental defects of long bones. *Clin Orthop Rel Res* 1992;277:229–237.
4. Auer JA, Watkins JP. Treatment of radial fractures in adult horses: an analysis of 15 clinical cases. *Equine Vet J* 1987;19:103–110.
5. Sanders-Shamis M, Bramlage LR, Gable AA. Radius fractures in the horse: a retrospective study of 47 cases. *Equine Vet J* 1986;18:432–437.
6. Crawford WH, Fretz PB. Long bone fractures in large animals. A retrospective study. *Vet Surg* 1985;14:295–302.
7. Hance SR, Bramlage LR, Schneider RK, et al. Retrospective study of 38 cases of femur fractures in horses less than one year of age. *Equine Vet J* 1992;24:357–363.
8. Letts RM, Afifi A, Sutherland JB. Technetium bone scanning as an aid in the diagnosis of atypical acute osteomyelitis in children. *Surg Gynecol Obstet* 1975;140:899–902.
9. Munoz P, Bouza E. Acute and chronic osteomyelitis and prosthesis-related infections. *Baillieres Best Pract Res Clin Rheumatol* 1999;13:129–147.
10. Abiri MM, DeAngelis GA, Kirpekar M, et al. Ultrasound detection of osteomyelitis. *Invest Radiol* 1992;27:111–113.
11. Reef VB, Reimer JM, Reid CF. Ultrasonographic findings in horses with osteomyelitis, in *Proceedings*. 37th Annu Meet Am Assoc Equine Pract 1991;381–391.
12. Russell RG. The assessment of bone metabolism in vivo using biochemical approaches. *Horm Metab Res* 1997;29:138–144.
13. Bettica P, Moro L, Robins SP, et al. Bone-resorption markers galactosyl hydroxlysine, pyridinium crosslinks, and hydroxyproline compared. *Clin Chem* 1992;38:2313–2318.
14. Garnero P, Shih WJ, Gineyts E, et al. Comparison of new biochemical markers of bone turnover in late postmenopausal osteoporotic women in response to alendronate treatment. *J Clin Endocrinol Metab* 1994;79:1693–1700.
15. Bollen AM, Kiyak HA, Eyre DR. Longitudinal evaluation of a bone resorption marker in elderly subjects. *Osteoporos Int* 1997;7:544–549.
16. Turner AS, Alvis M, Myers W, et al. Changes in bone mineral density and bone-specific alkaline phosphatase in ovariectomized ewes. *Bone* 1995;17:395S–402S.
17. Kurdy NM. Serology of abnormal fracture healing: the role of PIIINP, PICP, and BsALP. *J Orthop Trauma* 2000;14:48–53.

18. Lian JB, Gundberg CM. Osteocalcin: biochemical considerations and clinical applications. *Clin Orthop* 1988;226:267–271.

19. Price PA, Poser JW, Raman N. Primary structure of the gamma carboxy glutamic acid containing protein from bovine bone. *Proc Natl Acad Sci U S A* 1976;73:3374–3375.

20. Nyman MT, Paavolainen P, Forsius S, et al. Clinical evaluation of fracture healing by serum osteocalcin and alkaline phosphatase. *Ann Chir Gynaecol* 1991;80:289–293.

21. Ohishi T, Takahashi M, Kushida K, et al. Changes in biochemical markers during fracture healing. *Arch Orthop Trauma Surg* 1998;118;126–130.

22. Obrant KJ, Merle B, Bejui J, et al. Serum bone-gla protein after fracture. *Clin Orthop* 1990;258:300–303.

23. Bowels SA, Kurdy N, Davis AM, et al. Serum osteocalcin, total and bone-specific alkaline phosphatase following isolated tibial shaft fracture. *Ann Clin Biochem* 1996;33:196–200.

24. Mallmin H, Ljunghall S, Larsson K. Biochemical markers of bone metabolism in patients with fracture of the distal forearm. *Clin Orthop* 1993;295:259–263.

25. Ingle BM, Hay SM, Bottjer HM, et al. Changes in bone mass and bone turnover following distal forearm fracture. *Osteoporo Int* 1999;10:399–407.

26. Oni OO, Mahabir JP, Iqbal SJ, et al. Serum osteocalcin and total alkaline phosphatase levels as prognostic indicators in tibial shaft fractures. *Injury* 1989;20:37–38.

27. Emami A, Larsson A, Petrén-Mallmin M, et al. Serum bone markers after intramedullary fixed tibial fractures. *Clin Orthop* 1999; 368:220–229.

28. Peters KM, Rosendahl T, Heller KD, et al. Osteocalcin levels in chronic osteomyelitis. *Arch Orthop Trauma Surg* 1994;114:53–55.

29. Philipov JP, Pascalev MD, Aminkov BY, et al. Changes in serum carboxyterminal telopeptide of type I collagen in an experimental model of canine osteomyelitis. *Calcif Tissue Int* 1995;57:152–154.

30. Raekallio J, Mäkinen PL. Alkaline and acid phosphatase activity in the initial phase of fracture healing. *Acta Pathol Microbiol Scand* 1969;75:415–422.

31. Volpin G, Rees JA, Ali SY, et al. Distribution of alkaline phosphatase activity in experimentally produced callus in rats. *J Bone Joint Surg Br* 1986;68:629–634.

32. Bowles SA, Kurdy N, Davis AM, et al. Changes in serum bone-specific alkaline phosphatase following tibial fracture. *Ann Clin Biochem* 1997;34:690–691.

33. Leung KS, Fung KP, Sher AH, et al. Plasma bone-specific alkaline phosphatase as an indicator of osteoblastic activity. *J Bone Joint Surg Br* 1993;75:288–292.

34. Joerring S, Krogsgaard M, Wilbek H, et al. Collagen turnover after tibial fractures. *Arch Orthop Trauma Surg* 1994;113:334–336.

35. Wichmann MW, Arnoczky SP, DeMaso CM, et al. Depressed osteoblast activity and increased osteocyte necrosis after closed bone fracture and hemorrhagic shock. *J Trauma* 1996;41:628–633.

36. Delmas PD. Biochemical markers of bone turnover. *J Bone Miner Res* 1993;8:S549–S555.

37. Baltzer AW, Lattermann C, Whalen JD, et al. A gene therapy approach to accelerating bone healing. Evaluation of gene expression in a New Zealand white rabbit model. *Knee Surg Sports Traumatol Arthrosc* 1999;7:197–202.