Chapter 5

The Efficacy of the Bone Markers Osteocalcin and Carboxyterminal Cross-linked Telopeptide of Type I Collagen in Evaluating Osteogenesis in a Canine Crural Lengthening Model

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The Veterinary Journal 2006: in print

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Abstract

The aim of the present study was to determine the efficacy of the bone markers osteocalcin (OC) and carboxyterminal cross-linked telopeptide of type I collagen (ICTP) in evaluating new bone formation in the dog, using commercially available immunoassay kits. Dogs were randomly divided into three groups and a circular external skeletal fixation system (CESF) was mounted on the tibia. In the first group a distraction osteogenesis procedure of the crus was performed. The second group received an osteotomy without crural lengthening, whereas the third group served as a sham-operated control. Bone formation was assessed, using densitometric image analysis of crural radiographs. Despite significant differences in the amount of newly formed bone, this finding was not reflected in the plasma levels of OC and ICTP. In conclusion, OC and ICTP were not efficacious as markers of bone formation and resorption during osteogenesis in this canine model.

Introduction

Bone metabolism can be monitored non-invasively using markers of bone formation and resorption. A variety of commercially available bone marker assays has been developed. Although designed initially for use in humans, several of these assays are also validated to monitor bone metabolism in laboratory animals, dogs and horses.[8,9,10,25]

Bone markers can be subdivided into enzymatic markers and metabolic products of bone formation and resorption. The enzymatic markers include bone-specific alkaline phosphatase (BAP), an osteoblast related marker of bone formation, and tartrate-resistant acid phosphatase (TRAP), an osteoclast-related marker of bone resorption.[12,19,22] Serum or plasma markers of metabolic products of bone metabolism in companion animals include osteocalcin (OC), the carboxyterminal propeptide of type-I procollagen (PICP), the aminoterminal propeptide of type-I procollagen (PINP), the cross-linked carboxyterminal telopeptide of type-I collagen (ICTP), and the C-terminal cross-linked telopeptide of type-I collagen (CTX). OC is an osteoblast-related marker of bone formation PICP and PINP are markers of type-I collagen synthesis and hence bone formation.[11,14,38]

ICTP and CTX are markers of type-I collagen breakdown and hence bone resorption.[30] ICTP is released through the actions of matrix metalloproteinases and is therefore also known as CTX-MMP. CTX is released through he actions of cysteine proteinases, including cathepsin K.[20]

Bone markers have been used successfully in humans to monitor metabolic bone disease, including chronic renal disease, osteoporosis, hyperthyroidism, rheumatoid arthritis and growth hormone deficiency.[1,18,30,38,43,46] In the dog, reports concerning bone markers to monitor bone pathology or osteogenesis are limited to OC, ICTP, and BAP.[15,16,26,37]

In both man and dog, distraction osteogenesis is used to treat a variety of skeletal conditions, including bone length deficits, growth deformities, bone loss after trauma or radical resection, and cosmetic craniofacial surgery. [4,31,42,47] This principle allows the production of large quantities of new bone from the osteotomy sites under controlled mechanical distraction. In the dog, there is extensive experience with distraction osteogenesis, both clinically and as an animal model, although insights in the fundamentals of this type of bone formation are still insufficient. Both in research and clinical settings evaluation of the distraction bone regenerate is mainly performed by plain radiography, sonography, and the histomorphometric assessment of bone biopsies.[6,7,13,18,35,40,41] Application of advanced imaging techniques, including magnetic resonance imaging and

computed tomography, is often impeded by the presence of the external skeletal fixation system, used for the distraction procedure.

Bone markers could provide us with another means to evaluate bone formation and resorption during both clinical and experimental lengthening procedures and other types of bone healing. The objective of the present study was to determine using a canine crural lengthening model whether the bone markers OC and ICTP could be of value in evaluating osteogenesis following either a distraction procedure or bone healing after an osteotomy.

Materials and Methods

Animals

The Utrecht University Ethics Committee for Animal Care and Use approved all procedures in this study. The data presented in this manuscript were collected during a study, assessing the fundamentals of distraction osteogenesis, including the local expression of bone growth factors and systemic induction of growth factors.

Twelve mature Labrador retriever dogs were used, with a mean age of 20 months (range, 12 - 31 months), and a mean body weight of 27 kg (range, 21 - 32 kg). The animals were allocated to three groups. Dogs were fed a balanced commercial dog food twice a day at a set time (i.e., 0900 and 1800 h) and water was available ad libitum.

Surgery and Distraction

Circular External Skeletal Fixation (CESF) systems were prepared prior to surgery and steam sterilized (Imex Veterinary Inc., Longview, TX, USA). All frames were identical and consisted of two proximal and two distal full rings with a 100 mm diameter, connected by three treaded rods with a one mm pitch.

The dogs received medetomidine (Domitor, Pfizer Animal Health B.V., Capelle a/d IJssel, The Netherlands) as a pre-anaesthetic sedative and anaesthesia was induced IV with propofol (Rapinovet, Schering-Plough Animal Health N.V., Bruxelles, Belgium). General inhalation anaesthesia was accomplished with nitrous oxide, oxygen, and isoflurane. Amoxicillin with clavulanic acid (Augmentin, SmithKline Beecham Farma B.V., Rijswijk, The Netherlands) was administered IV (20 mg/kg BW) prior to surgery.

The right hind limb was prepared in a standard sterile fashion. The CESF was placed on the right tibia with the distal ring 3 cm proximal of the tarsocrural

joint. The most proximal and distal rings were mounted on the tibia, using two 1.5 mm diameter transosseus wires each. The two inner rings were both mounted to the tibia with one 1.5 mm diameter transosseus wire. All wires were tensioned, using a dynamometric wire tensioner (Hofmann SaS, Monza, Italy), to an equivalent of 60 kg. An anteromedial approach to the tibia and fibula was used. The periosteum of the tibia was preserved by making a longitudinal incision of 3 cm and by carefully elevating the periosteum circumferentially from the bone. In the dogs of the distraction (n=4) and osteotomy (n=4) group the tibia and fibula were osteotomized in the diaphysis, while protecting the periosteum, at two-thirds of the tibial length from the proximal, using an oscillating saw and ample lavage for cooling. In the dogs of the control group (n=4), after placement of the CESF, the periosteum of the tibia was also elevated. In all three groups the periosteum was closed with an absorbable suture material and closure of the subcutis and skin was in a routine fashion.

A protective full-leg bandage was applied for 3 days. There after, the CESF was protected only with a bandage. The dogs received analgesics (buprenorphine, 4dd 10 μg/kg BW) for 3 days post operatively. Full loading of the legs was permitted immediately after surgery. Dogs were housed in cages for the duration of the study. After a 4-day latency, distraction was started with a rate of 0.5 mm twice a day for a period of 10 consecutive days in the distraction group. All dogs were euthanatized with an overdose of barbiturates at 6 weeks after surgery.

Collection of plasma samples

Jugular vein blood samples were collected into EDTA tubes in the week prior to surgery (week 0) to obtain preoperative values. The samples were centrifuged for 15 min at 3.4 g and plasma was collected into plain tubes and stored at -20°C until analysis. As we were interested in bone consolidation following the lengthening procedure, plasma samples were then collected once a week, starting at the end of the distraction period (i.e. week 2) for five consecutive weeks. All blood samples were taken at the same time of the day (i.e., 0800 h).

Assay for osteocalcin (OC)

Plasma OC concentrations were determined, using a commercially available intact human osteocalcin ELISA kit validated for the dog and following the manufacturer's instructions (Biomedical Technologies Inc, Stoughton, MA, USA).[16] The assay measures only intact osteocalcin and it eliminates any potential confounding interference by circulating fragments. The assay is a sandwich ELISA, which utilises monoclonal antibodies directed towards the amino- and

carboxy-terminal regions of the protein. The inter- and intra-essay coefficient of variability (CV) were 10.5% and 7%, respectively, with a sensitivity of 0.5 µg/L (unpublished data).

Assay for carboxyterminal cross-linked telopeptide of type-I collagen (ICTP)

Plasma ICTP concentrations were determined using a commercially available UniQ ICTP RIA kit validated for the dog and following the manufacturer's instructions (Orion Corp. Orion Diagnostica, Espoo, Finland). This RIA kit is based on the competitive radioimmunoassay technique. A known amount of labelled ICTP and an unknown amount of unlabelled ICTP in the same sample compete for the limited number of high affinity binding sites of the rabbit anti-ICTP antibody. After separating the free antigen, the amount of labelled ICTP in the sample tube is inversely proportional to the amount of ICTP in the sample. Serial dilutions of canine plasma resulted in a curve parallel to the standard curve of human ICTP. The inter- and intra-assay CVs were 5.7% and 4.9% at 6.1 µg/L, and 4.1% and 4.7% at 32 µg/L, respectively, with a sensitivity of 0.7 µg/L.[44]

Radiography and densitometry

Immediately after surgery standardized radiographs of the right crus in all three groups in a posterior-anterior (PA) and lateromedial (LM) direction were obtained and on a weekly basis thereafter to follow-up bone formation in the distraction and osteotomy zone. The radiographs of the CESF and right tibia included a ruler and an aluminium step-wedge, consisting of a total of ten 2 mm thick aluminium slabs mounted in an overlapping manner. Bone formation was quantified using a densitometric image analysis system at 5 and 6 weeks post-surgery.[16]

Radiographs were recorded with a Sony b/w CCD camera type XC-77CE and digitized for image analysis (frame size 752 x 574 pixels; 256 grey levels) with a PC-based system equipped with the KS400 version 3.0 software (Carl Zeiss Vision, Oberkochen, Germany). A program was developed in KS400 to quantify the amount of regenerated bone. Each radiograph of the bone was calibrated geometrically and densitometrically, using the image of a ruler and the aluminium step-wedge. The densitometric calibration was performed by measuring the mean optical density of a square area of 50 x 50 pixels in six steps of the aluminium model (0,2,4,6,8, and 10 mm). The measurement was carried out in a median filtered image to reduce the influence of the photographic grains in the film.

The optical density values are a polygonal fit with the aluminium values to produce a transformation table, which enables the amount of newly formed bone to

be express in equivalents of cubic mm of aluminium. The region of interest (ROI) was centred over the distraction or osteotomy zone and included all new bone formation. The ROI was delineated on the digitalized PA and LM images and densitometric analyses for bone area and bone amount were performed. The means of the PA and LM bone area and bone density were determined for statistical analysis.

Statistical analysis

OC and ICTP plasma concentrations were evaluated using an ANOVA for repeated measures and a Tukey HSD post hoc test. Densitometric data, including the bone area and bone amount, and the OC and ICTP concentrations within the groups were compared using a Student's *t* test. Correlations between the bone markers OC and ICTP and the densitometric data were examined, using the Pearson correlation test. *P* values < .05 were considered significant. All data analyses were performed using the SPSS 10.1 statistical package (SPSS Inc, Chicago, IL, USA).

Results

Plasma concentrations of OC

During the postoperative period, OC plasma concentrations tended to be lower than pre surgery in all three groups. In both the distraction and osteotomy group plasma OC concentrations were significantly ($P < .05$) lower than preoperative values from week 2 to 5. Although the decline in OC plasma concentrations was most pronounced in the distraction group, no significant differences were demonstrated between the groups at any time during the study (Fig 1).

Plasma concentrations of ICTP

A significant difference ($P < .05$) in the ICTP plasma concentrations was found between the control group and the osteotomy group only at 2 weeks after surgery. The ICTP plasma concentrations within the groups did not differ from the preoperative levels. In the distraction and osteotomy group ICTP concentrations did not change during the phase of bone formation (Fig 2).

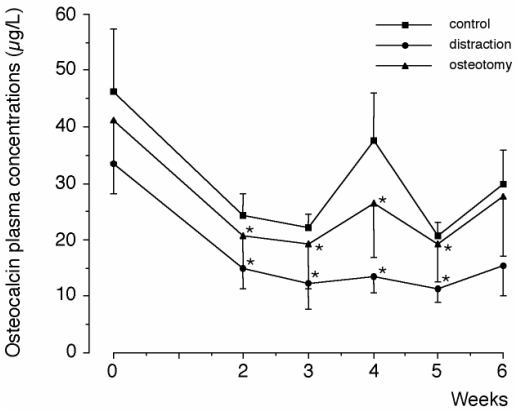

Fig 1. Osteocalcin (OC) plasma concentrations of the control, distraction, and osteotomy group during the 6-week study period. Data are presented as the mean ± SE. * Significant difference in comparison with the preoperative values at week 0 ($P < .05$).

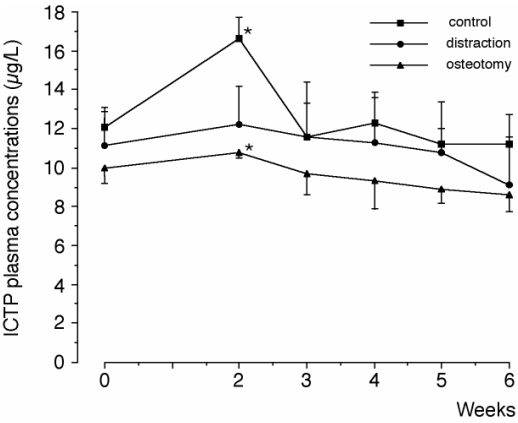

Fig 2. Carboxyterminal cross-linked telopeptide of type I collagen (ICTP) plasma concentrations of the control, distraction, and osteotomy group during the 6-week study period. Data are presented as the mean ± SE. * Significant difference between the groups at that time ($P < .05$).

Radiography and densitometry

The distraction procedure was uneventful and no serious complications regarding the CESF were encountered. Superficial transosseus wire tract infection was the most common complication in all groups, but could be managed successfully with oral antibiotics and local wound care.[33]

Periosteal new bone adjacent to the site of osteotomy was already present as early as 1 week postoperatively in both the distraction and osteotomy group. The amount and density of the periosteal bone increased over time. In the distraction zone new bone formation occurred at 3 weeks postoperative and the periosteal

bone had merged with the bone in the distraction zone at 4 weeks. In the control group no periosteal bone formation was seen at the site of periosteal elevation.

At 5 weeks after surgery, the newly formed callus had mineralised to such an extent that densitometric assessment was possible. The densitometric evaluation revealed a significantly greater bone area and bone amount in the distraction group than in the osteotomy group both at 5 and 6 weeks after surgery. Bone formation as indicated by the bone area and bone amount was larger by more than a factor of 2 larger in the distraction group compared with the osteotomy group. Within the distraction group both the bone area and bone amount had increased significantly from week 5 to 6. In the osteotomy group this increase was only significant for the bone amount (Table 1).

Table 1. Densitometric image analyses of bone formation in the distraction (n=4) and osteotomy (n=4) group.

	Bone area (mm^2)		Bone amount (mm^3 Al x 10^3)	
	Week 5	Week 6	Week 5	Week 6
Distraction	$277.6 \pm 39.7^{*}$	$366.6 \pm 52.8^{*,\#}$	$278.0 \pm 50.7^{*}$	$365.6 \pm 67.0^{*,\#}$
Osteotomy	120.6 ± 18.8	149.0 ± 26.7	132.9 ± 24.5	$166.5 \pm 32.7^{\#}$

Data are presented as the mean ± SE. Bone amount is given in equivalents of Aluminium (Al) in mm^3 x 10^3. No new bone formation was found in the control group. * Significant differences between the two groups at the corresponding time ($P < .05$). # Significant increase within the group between weeks 5 and 6 ($P < .05$).

Correlations between the bone markers OC and ICTP and densitometry

No significant correlations were found between OC and ICTP at any time during the study. The results of the densitometric measurement, including the bone area and bone amount, showed no correlations with the plasma concentrations of either OC or ICTP.

Discussion

The specific aim of this study was to investigate, using a distraction osteogenesis model, whether commercially available OC and ICTP bone marker assays could be of value in monitoring bone formation and resorption during bone healing and whether they might therefore have clinical usefulness in dogs. Ideally, the bone markers should be able to monitor early bone formation, to assess progression of bone consolidation, and to predict the outcome of bone healing.

The discriminatory power of these assays depends on analytic variability and biological variability. The analytic variability is composed of variability within the assay and inter-laboratory variation.[45] Factors affecting the biological variability include age, sex, exercise and systemic disease.[25,27,36,48] In dogs, age has been shown to be an important biological factor of variation.[2,3] In the present study, we used skeletally mature dogs in the same age range which minimized the influence of this factor. ICTP levels have been reported not to differ depending on breed size of the dog.[8] Clear diurnal and seasonal variation has however been demonstrated in various bone markers, specifically OC and ICTP, both in humans and several animal species, including dogs, horses, rats, and mice.[21,25,28,29,34,39]

Standardizing feeding time and the time at which blood was sampled eliminated the diurnal variation of OC and ICTP. Ladlow et al. (2002) reported no significant variation of marker expression over a 12-week period.[25] This was twice the duration of the present study and long-term variation should not be a factor. An issue that cannot be easily resolved is the fact that the direct comparison of results between different studies is impeded by the inter-laboratory variation of bone marker assays.[45]

The radiographic data in this study demonstrated a successful lengthening procedure in analogy with previous models of distraction osteogenesis in both dogs and other species. During distraction osteogenesis bone is primarily produced through intramembranous ossification, whereas endochondral ossification is encountered during healing on an osteotomy.[4] Intramembranous ossification is characterized by direct formation of new bone. During endochondral ossification a cartilaginous callus is formed first, which is secondarily replaced by cancellous bone. Ideally, one would expect earlier and more pronounced alteration of OC plasma concentrations during distraction osteogenesis in comparison with osteotomy bone healing. As remodelling of the primarily cartilaginous callus and thus collagen resorption is essential during endochondral ossification, this could result in more pronounced effects on ICTP plasma concentrations. In addition, the difference in the amount of newly formed bone was expected to be another discriminatory factor. Unfortunately the bone markers OC and ICTP were not efficacious in demonstrating these differences in osteogenesis.

In the present study, OC plasma concentrations decreased and although no significant differences were found between the groups, the OC concentrations were lowest in the distraction group. This is interesting as these dogs showed the highest amount of bone formation. Ducy et al. (1996) demonstrated increased bone formation in OC-deficient mice, indicating a suppressive role of OC on bone formation, possibly through inhibition of osteopontin.[14] In view of this, decreased OC plasma concentrations could be consistent with increased bone formation. The decline in OC plasma concentrations in the control group could be explained by the stimulating effect of the CESF of crural blood flow and thus bone metabolism.[5] In contrast, Lammens et al. (1998) reported increased OC plasma levels during distraction osteogenesis in a dog study.[26] As the assay in the present study only measured intact OC, the difference could be explained by the fact that OC metabolites were included in the earlier study.[23] Whether OC metabolites are biologically active during osteogenesis or this process depends exclusively on intact OC, is not clear. In view of these considerations OC seems to be a questionable marker of bone formation in the dog.

In the present study an osteotomy instead of the technically more demanding corticotomy was performed, as experimental studies comparing both methods revealed no differences in the outcome of the distraction procedure.[13,17,24] Both in experimental and clinical settings, the use of an oscillating saw for the osteotomy in combination with ample lavage has been shown to be effective in dogs.[32] Nevertheless, enhanced ICTP levels were more likely to be expected in the distraction and osteotomy groups than in the control group. An osteotomy will cause some trauma to the bone, which is expected to initially increase bone resorption. In addition, formation of new bone proceeds with both accretion and resorption of collagen and thus should increase ICTP levels. In contrast, we only found a significant difference between the osteotomy and control group at 2 weeks after surgery. The rise in ICTP levels within the control group at 2 weeks post surgery can only be explained as the result of a reaction to the transosseus wires of the CESF. Although there were no radiographic or clinical indications for a local osteomyelitis, enhanced bone resorption at the bone-wire interface could contribute to higher ICTP levels.[37] In the distraction and osteotomy group active bone formation was already present at 2 weeks after surgery with collagen accretion surmounting collagen degradation. As ICTP is released through the actions of MMP, a difference in the expression of MMP in the control group, shifting the equilibrium initially towards collagen degradation, could be responsible for this finding. As we did not measure ICTP levels during the first week after surgery, we could not assess the initial influence of the CESF on ICTP plasma concentrations.

In view of the previous observations, it was not surprising that no correlations were found between OC and ICTP and between these markers and the amount of newly formed bone.

In conclusion, the clinical utility of the commercial assays for the bone markers OC and ICTP in evaluating bone formation related to distraction osteogenesis and osteotomy bone healing is questionable in the dog. Nevertheless, combinations of other bone markers may be efficacious in monitoring new bone formation in dogs and should be explored in more detail.

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