Chapter 4

Expression of Osteotropic Growth Factors and Growth Hormone Receptor in a Canine Distraction Osteogenesis Model

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Abstract

Osteotropic growth factors play an important role in bone metabolism. Nevertheless, knowledge about their expression in relation to distraction osteogenesis remains limited. The aim of the present study was to determine the expression of growth hormone (GH), growth hormone receptor (GHR), insulin-like growth factor-I (IGF-I), insulin-like growth factor-II (IGF-II), and bone morphogenetic protein-2 (BMP-2) in distraction-induced bone regenerate. Expression of these factors was assessed during the consolidation phase, comparing distraction osteogenesis with osteotomy-induced bone formation. Real time PCR was performed as a semi-quantitative measurement of mRNA and the relative expression levels of these factors were determined. In addition, plasma GH profiles and plasma concentrations of IGF-I, IGF-II, and insulin-like growth factor binding protein- 4 and -6 (IGFBP-4 and -6) were measured to assess their potential systemic role during bone formation. Expression of GHR, IGF-I, and BMP-2 had significantly increased in comparison with the expression of these factors in mature bone. Expression of GHR was significantly higher in distraction-induced bone regenerate than in osteotomy-induced bone. No significant differences were found for the expression of IGF-I and BMP-2 between distraction and osteotomy. Plasma concentrations of GH, IGF-I, IGF-II, IGFBP-4, and IGFBP-6 did not demonstrate any significant differences between treatment groups and controls. Up-regulation of GHR expression in distraction osteogenesis may enhance sensitivity to endogenous systemic GH and thus promote consolidation of the bone regenerate. Changes in the systemic osteotropic growth factors GH, IGF-I, IGF-II, IGFBP-4, and IGFBP-6 do not seem to be of importance during distraction osteogenesis.

Introduction

Distraction osteogenesis is a widely used method in limb lengthening, deformity correction, bone segment transport, and cosmetic craniofacial surgery. The principle allows for the formation of new bone following slow distraction of an osteotomy.[23,24] Although the radiographic, histological and ultra-structural changes associated with this process have been delineated, knowledge about the interactions of the growth factors governing this process is still insufficient.[1,38,42] In accordance with bone fracture models, the expression of several osteotropic growth factors, including insulin-like growth factor-I (IGF-I), transforming growth factor-β (TGF-β), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and bone morphogenetic proteins (BMP) has been demonstrated in distraction osteogenesis.[12,14,31,40,52] Growth hormone (GH), IGF-I and insulin-like growth factors-II (IGF-II) play a critical role during bone growth and bone accretion.[35] The actions of IGF-I and -II are modulated through their six high-affinity binding proteins (IGFBPs).[34] In general, IGFBP-1, -2, -4, and -6 inhibit and IGFBP-3 and -5 stimulate osteoblast function.[26,34] More recently, focus has been on angiogenesis and the role of vascular endothelial growth factor (VEGF) and VEGF receptor-1 and -2 (VEGFR-1 and -2).[5] Osteotropic and angiogenic factors, including GH, IGF-I, TGF-β, BMP-4, FGF-2, and VEGF have been used experimentally to stimulate bone formation during distraction osteogenesis.[10,13,28,37,39,41,47] Aim of these studies was to shorten the protracted treatment period, required for lengthening and subsequent consolidation of the bone regenerate in the clinical situation.

During distraction osteogenesis osteotropic and angiogenic factors are produced which exert their effect both locally and systemically.[18,22,25,30,51] In view of the complex interactions of the osteotropic factors and close relations between GH, GHR, IGFs, and IGF binding proteins (IGFBP) we decided to focus on the GH-IGF axis and include BMP-2 as a potent stimulator of osteogenesis.[43] Aim of the present study was to assess the local and systemic role of GH, GHR, IGF-I, IGF-II, IGFBP-4 and IGFBP-6 in distraction osteogenesis. Densitometric image analysis was used to quantify the amount of newly formed bone.

Materials and Methods

Animals

The Utrecht University Ethics Committee for Animal Care and Use approved all procedures in this study. Eighteen mature Labrador retriever dogs

were used, including 14 females and 4 males, with a mean age of 19 months (range; 12 - 31 months), and a mean body weight of 26 kg (range; 21 - 32 kg). The dogs were allocated to three groups of 6 animals each. A standard commercial dog food was fed twice a day and water was available ad libitum. This feeding regime was continued throughout the entire study and food was not withheld prior to or during blood sample collection. The body weight of the dogs was determined once a week to assess potential weight loss.

Collection of blood plasma samples

Jugular vein blood samples were collected into ice-chilled EDTA tubes and kept on ice prior to centrifugation for 15 min at 3.9 g and 4°C, within 60 min after collection. Plasma samples were stored at -20°C until analysis. GH profiles for each individual animal were determined by collecting plasma samples every 15 minutes over an 8-hour period in the week prior to surgery and during the first, second, fourth, and sixth week after surgery. Plasma samples of IGF-I, IGF-II, IGFBP-4, and IGFBP-6 were obtained twice per week, starting in the week prior to surgery for the duration of the study. To rule out diurnal variations all blood samples were taken at the same time of the day (i.e. 0800 hours).

Surgery and distraction

In all dogs, a circular external skeletal fixation system (CESF) was applied to the right tibia (Imex Veterinary Inc., Longview, TX, USA) in all dogs. 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 CESF was assembled prior to surgery and steam sterilized.

The dogs received medetomidine (Domitor®, Pfizer Animal Health B.V., Capelle a/d IJssel, The Netherlands) as a pre-anesthetic sedative and anesthesia was induced intravenously with propofol (Rapinovet®, Schering-Plough Animal Health N.V., Bruxelles, Belgium). General inhalation anesthesia was accomplished with nitrous oxide, oxygen, and isoflurane. Amoxicilline with clavulanic acid (Augmentin®, SmithKline Beecham Farma B.V., Rijswijk, The Netherlands) was administered intravenously (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 crus and the proximal and distal ring were attached to the tibia, using two 1.5 mm diameter transosseus wires, whereas a single 1.5 mm diameter transosseus wire was used for the central rings. All wires were tensioned with a dynamometric tensioner (Hofmann SaS, Monza, Italy), to an equivalent of 60 kg. After elevating

the periosteum, the tibia and fibula in the distraction (n=6) and osteotomy (n=6) group were osteotomized with an oscillating saw and ample lavage for cooling. In the control dogs (n=6) the periosteum was also elevated after placement of the CESF. The periosteum was closed with an absorbable suture material and closure of the subcutis and skin was in a routine fashion. A protective bandage was applied for 3 days. The dogs received buprenorphine (Temgesic®, Schering-Plough B.V., Utrecht, The Netherlands) as a postoperative analgesic (4dd 10 μg/kg bw) for three days. Full weight bearing of the legs was permitted immediately after surgery. In the distraction group, lengthening was started after a 4-day latency at a rate of 0.5 mm twice a day for a period of 10 consecutive days.

Biopsies of the distraction, osteotomy, or control zone were taken at the right side under general anesthesia in one dog per group at 2 and 4 weeks after surgery, respectively. These biopsies were immediately frozen in liquid nitrogen, stored at –70° C and used for mRNA isolation to test the primers for the PCR amplification (data not shown). All dogs were euthanatized with an overdose of barbiturates at 6 weeks after surgery. Segmental biopsies of the right and left tibia-fibula with a length of 3 cm, including the distraction, osteotomy, or control zone were obtained and spit longitudinally. One halve was immediately frozen in liquid nitrogen and stored at –70° C until required for RNA isolation, while the other halve was processed for histological examination (data not shown).

Biochemical analysis

Plasma GH concentrations were measured in a homologous RIA as previously described.[11] The Pulsar program developed by Merriam and Wachter was used to analyze the GH profiles.[32] The values extracted from this program included the mean of the smoothed baseline (GH-B), the area under the curve (AUC) for GH above the zero-level (GH-AUC-0), and the AUC for GH above the baseline (GH-AUC-B).

Total plasma IGF-I and IGF-II concentrations were measured after acid-ethanol extraction to remove interfering IGFBPs.[15] The absence of interfering IGFBPs was confirmed according to the protocol of Sota *et al.*[45] IGF-I concentrations were measured in a heterologous RIA validated for the dog.[36] IGF-II concentrations were determined with a heterologous RIA, using monoclonal antibodies against rat IGF-II (Amano Enzyme U.S.A. Co., Lombard, IL, USA) as described previously.[15]

Plasma IGFBP-4 and IGFBP-6 concentrations were determined with a specific heterologous RIA, using polyclonal antiserum WKZ8209 and WKZ6278,

respectively.[48,49] The mean concentration of the in duplicate plasma samples was determined for each week.

Gene expression

Frozen bone regenerate was ground in liquid nitrogen pre-frozen cups of a micro-dismembrator (Micro-Dismembrator U, B. Braun Biotech International GmbH, Melsungen, Germany). Five hundred milligram of milled bone tissue was resuspended in Qiagen lysis buffer (Qiagen GmbH, Hilden, Germany) and centrifuged for 10 min at 5 *g*. The supernatant was applied to a Qiagen midi-column (Qiagen GmbH, Hilden, Germany) and total RNA was isolated according to the manufacturer's protocol. After DNase I treatment (DNA*free*™ kit, Ambion, Austin, TX, USA) the isolated total RNA (130 ± 58 ng; mean ± SEM) was amplified, using the SMART™ PCR cDNA synthesis protocol (Clontech Laboratories, Inc., Palo Alto, CA, USA). The yield of SMART™ cDNA was 2.2 ± 0.2 µg out of 25 ng total RNA. SMART™ cDNA was purified by use of silica-gel membrane spin-columns (QIAquick PCR Purification kit, Qiagen GmbH, Hilden, Germany) and brought to a concentration of 1 ng/µl in water.

Real time PCR based on the high affinity double-stranded DNA-binding dye SYBR green I was performed in triplicate in a spectrofluorimetric thermal cycler (iCycler, BioRad, Hercules, CA, USA). Data were collected and analyzed with the provided application software. In addition, except for BMP-2, intron-spanning primer pairs were designed. β-Actin was used as endogenous reference. In order to confirm that β-actin levels were not influenced by the experimental manipulation and to further rule out interference of β-actin pseudogenes, GAPDH and β-actin, using a primer pair that distinguishes cDNA from pseudogene DNA, were also tested as internal standards.

For each real time PCR reaction, 10 ng of SMART™ cDNA was used in a reaction volume of 50 µl, containing 1x PCR buffer, 3 mM $MgCl_2$, 1:100,000 dilution of SYBR® green I (BMA, Rockland, ME, USA), 10 nM fluorescein calibration dye (BioRad, Hercules, CA, USA), 200 µM dNTPs, 20 pmol forward primer, 20 pmol reverse primer, and 1.25 units of AmpliTaq Gold DNA polymerase (Applied Biosystems, Roche, Branchburg, NJ, USA). Cycling conditions were 5 min at 95° C followed by 45 cycles of 15 sec at 95° C, 30 sec at 60° C, and 40 sec at 72° C. Primer pairs were designed, using PrimerSelect software (DNASTAR Inc., Madison, WI, USA) (Table 1).

Melt curves (iCycler) and agarose gel electrophoresis were used to examine each sample for purity and standard sequencing procedures (ABI PRISM™ 310 Genetic Analyzer, Applied Biosystems, Foster City, CA, USA) were

used to verify the analytical specificity of the PCR products. The individual melting curves proved that a single, unique product was amplified. Standard curves constructed by plotting the log of starting amount versus the threshold cycle were generated using serial 10-fold dilutions of known amounts of PCR products (from a conventional PCR). The amplification efficiency, E (%) = $(10^{(1/-s)} - 1) \times 100$ (s = slope), of each standard curve was determined and appeared to be >90% over a large dynamic range (6-8 orders of magnitude). Serial dilutions of SMART cDNA were also tested resulting in similar E values over 2-4 orders of magnitude and optimal input values of 10 ng of cDNA per reaction.

Table 1. Primer pairs used in the PCR amplification of cDNA generated from total mRNA in the various bone biopsies.

Gene	Primer (5' – 3')	Exon	Amplicon length (bp)
β-actin	F: TGGCACCACACCTTCTACAACGAG	3	180
	R: AGAGGCATACAGGGACAGGACAGC	4	
BMP-2	F: CAGAAATGAGTGGGAAAACAAC	3	207
	R: GTCTGGTCACGGGGAACTT	3	
IGF-I	F: ATGTCCTCCTCGCATCTCTT	3	355
	R: TCCCTCTACTTGCGTTCTTC	5	
IGF-II	F: CGCAGCCGTGGCATCGTTGAGGAG	5	200
	R: CTGCGCAGGCGCTGGGTGGACT	6	
GH	F: CCTGATGCGGGAGCTGGAAGATG	4,5	130
	R: GAAGCAGGAGAGCAGCCCGTAGTT	5	
GH-R	F: GATCCACCCATTGGCCTC	6	471
	R: AATCTTTGGAACTGGAACTGGG	9	

For each experimental sample the amount of target (GH, GHR, IGF-I, IGF-II, and BMP-2), with β-actin and GAPDH as endogenous references, was determined from the appropriate standard curve. The amount of target was divided by the amount of endogenous reference to obtain a normalized target value. Each of the normalized target values was divided by the normalized target value of the calibrator (osteotomy group) to generate *n*-fold relative expression levels.

Densitometric image analysis

Densitometric image analysis was used in order to quantify the amount of bone regenerate and to evaluate the distraction procedure. Immediately after surgery standardized radiographs of the right crus were obtained in a caudocranial (CdCr) and lateromedial (LM) direction and on a weekly basis thereafter. The radiographs included two rulers and an aluminum step-wedge, consisting of a total of ten 2 mm thick aluminum slabs mounted in an overlapping manner. 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 gray 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. Geometric and densitometric calibration of each radiograph was performed, using the ruler and aluminum step-wedge images. Densitometric calibration was executed by measuring the mean optical density of a square area of 50 x 50 pixels in 6 steps of the aluminum model (0,2,4,6,8, and 10 mm). The optical density values supplied a polygonal fit with the aluminum values to produce a transformation table, which enabled to express the amount of newly formed bone in equivalents of cubic mm of aluminum. The bone regenerate was delineated on the digitalized CdCr and LM images and densitometric analyses for mean bone area and mean bone amount were performed.

Statistical analysis

Real time PCR data were evaluated, using the Student's *t*-test for analysis of the log-transformed normalized target values. Growth factor data were evaluated with a general linear model for repeated measures and a paired sample T-test. Bone area and bone amount were evaluated using a Student's *t*-test and a paired samples T-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

Biochemical analysis

The GH profiles of the dogs were very similar and demonstrated no significant differences between the groups at any time. Prior to surgery, GH-B, GH-AUC-0, and GH-AUC-B levels were 1.4 ± 0.3, 23.8 ± 3.6, and 11.5 ± 2.0,

respectively (mean ± SEM). The cumulative means of GH-B, GH-AUC-0, and GH-AUC-B from week 1 to 6 in the distraction group were 1.5 ± 0.2, 25.2 ± 2.7, and 13.2 ± 1.8; in the osteotomy group 1.9 ± 0.3, 29.1 ± 3.2, and 14.0 ± 1.9; and in the control group 1.5 ± 0.2, 28.6 ± 2.4, and 16.4 ± 2.2, respectively.

The IGF-I plasma concentrations revealed a significant decrease in the first weeks following surgery, which was similar in magnitude in all groups (Fig. 1A). IGF-II and IGFBP-4 plasma concentrations remained relatively constant during the experimental period and did not differ between groups (Fig. 1B and 1C). IGFBP-6 plasma concentrations demonstrated a decline in comparison with pre-operative levels, which was most pronounced in the distraction group (Fig. 1D).

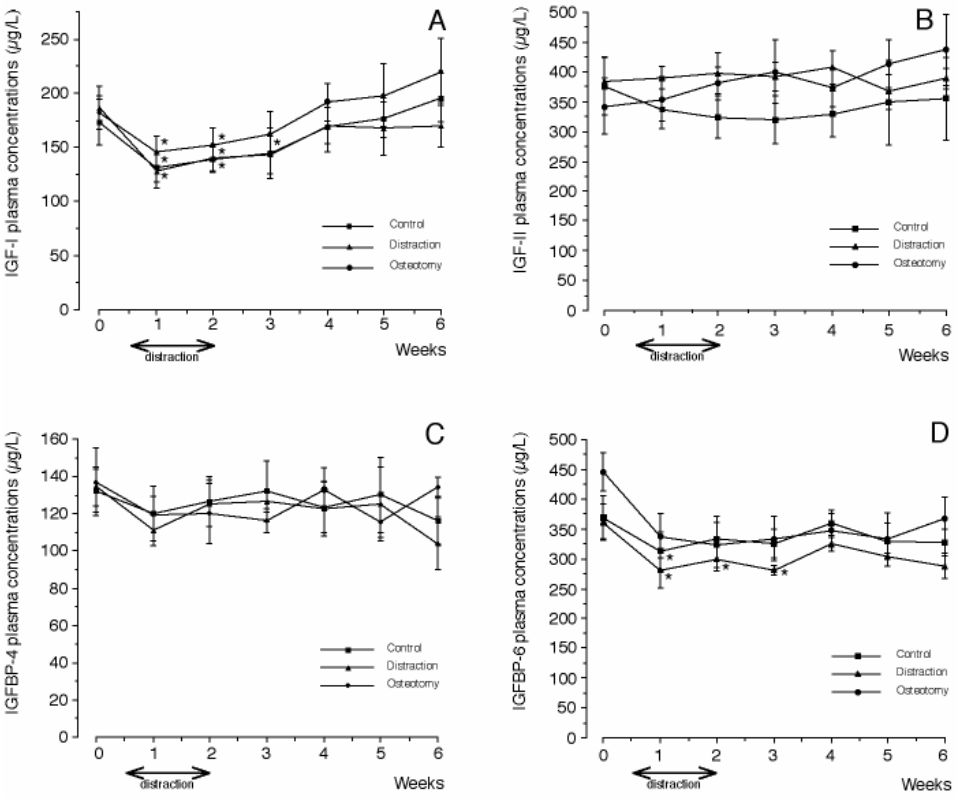

Figure 1. IGF-I, IGF-II, IGFBP-4, and IGFBP-6 plasma concentrations in the control, distraction, and osteotomy group during the 6-week study period.
Data are presented as the mean ± SEM. Week 0 shows the pre-operative plasma concentrations.
* indicates significant difference in comparison with preoperative levels of week 0 ($P < .05$).

Table 2. Normalized target values of BMP-2, IGF-I, and GHR gene expression determined by real time PCR in biopsies of osteotomy- and distraction-induced bone regenerate.

Bone biopsy	BMP-2	IGF-I	GHR
Osteotomy			
Median	0.54	0.97	0.03
Range	0.11 - 0.71	0.44 - 2.36	0.01 - 2.90
n-Fold (Calibrator)	1	1	1
Distraction			
Median	0.18	1.53	2.34
Range	0.08 - 0.43	0.80 - 2.32	0.10 - 5.44
n-Fold	0.3	1.6	78*

n-Fold is the relative expression level of bone morphogenetic protein-2 (BMP-2), insulin-like growth factor-I (IGF-I), and growth hormone receptor (GHR) in the distraction-induced bone regenerate with the osteotomy bone regenerate serving as the calibrator. * indicates a significant difference ($P < .05$).

Gene expression

In the right and left bone biopsies of the control as well as in the left specimens of the distraction and osteotomy group, the expression levels of GH, GHR, IGF-I, IGF-II, and BMP-2 were hard to determine, due to very low amounts of total RNA. The mRNA levels of GH and IGF-II were insufficient for reliable measurements, in spite of the fact that both GH and IGF-II mRNA could be clearly detected, using cDNA prepared from a similar amount of total mRNA originating from juvenile bone, still containing the epiphyseal growth plate (data not shown). Expression of GHR, IGF-I, and BMP-2 had significantly increased in comparison with the expression of these factors in the control bone segments. Compared to the control values (data not shown), BMP-2 expression in the biopsies of the osteotomy group at week 6 was significantly increased (10-fold), whereas the rise in BMP-2 expression in the distraction group was less pronounced (5-fold).

Comparing the expression levels of BMP-2, IGF-I, and GHR in the distraction group with the osteotomy group (calibrator) demonstrated a significant

78-fold up-regulation of the relative expression level of GHR (Table 2). The differences in BMP-2 and IGF-I relative expression levels were not significant. Similar results were obtained when GAPDH and β-actin were used as endogenous references (results not shown).

Distraction and densitometric image analysis

The distraction procedure was uneventful and no serious complications were encountered. Weight loss was not observed in the post-surgical period. Periosteal new bone formation adjacent to osteotomy site was already present radiographically as early as one 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 was visible at 3 weeks postoperative and the periosteal bone had merged with the bone in the distraction zone at 4 weeks. Bridging of the osteotomy was not seen before 6 weeks after surgery in the osteotomy group. Periosteal elevation in the sham-operated control group did not lead to periosteal new bone formation.

Densitometry revealed a significantly larger bone area and bone amount in the distraction group in comparison with the osteotomy group both at 5 and 6 weeks after surgery. Within the distraction group both bone area and bone amount had increased significantly during this period (Table 3).

Table 3. Densitometric image analysis of the bone formation in the distraction and osteotomy 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 ± SEM. Bone amount is given in equivalents of Aluminum (Al) in mm^3 x 10^3. * Significant differences between groups at the corresponding time ($P < .05$). # Significant increase in time within the group ($P < .05$).

Discussion

In the present study, radiographic data and densitometric image analysis were consistent with a successful lengthening procedure in analogy with previous models of distraction osteogenesis in dogs.[9,23,24,30,38] Distraction osteogenesis is characterized by intramembranous ossification, whereas endochondral bone formation is encountered during healing of an osteotomy. This could lead to differences in the expression levels of GH, GHR, IGF-I, IGF-II, and BMP-2 in the bone regenerate. In addition, alterations in the local production of osteotropic growth factors could result in a shift of GH, IGF-I, IGF-II, IGFBP-4, and IGFBP-6 plasma concentrations.[30]

Expression of GH mRNA has been demonstrated in the metaphyseal areas adjacent to growth plate in dogs.[29] In the present study, GH expression in the bone regenerate was very low and did not seem to play an important role at this stage of bone formation. Although the role of GHR in the growth plate is slowly elucidated, very little is known about its role in distraction osteogenesis and bone healing.[8,17,27]. In the present study, GHR expression was also demonstrated in the osteotomy-induced callus, but there was a clear up-regulation in the distraction-induced bone regenerate. Mechanical tension-stress could be responsible for the increased GHR expression levels in the distraction-induced bone regenerate.[6] Raising the local sensitivity to GH may contribute in enhancing bone consolidation after distraction osteogenesis.[2,39] In the present study, up-regulation of GHR without alterations in IGF-I expression and IGF-I plasma concentrations could suggest a direct effect of endogenous GH on consolidation of the bone regenerate.[19,50]

Hypophyseal GH is presumed to represent the major constituent of GH in the circulation and GH release and IGF-I production are closely interconnected.[7,44] In the present study, changes in the GH 8-hour secretory profiles, including GH-B, GH-AUC-0, and GH-AUC-B, could not account for the initially decreased IGF-I plasma concentrations. Reports on IGF-I plasma levels during distraction osteogenesis have been contradictory and seem to be related to the duration and amount of lengthening.[4,30,33,51] In the present study, the initial decline in IGF-I plasma levels was similar in all groups. Although post-operative weight loss was excluded as a cause of decreased IGF-I production, a temporary relative insensitivity to GH due to stress could play a role in this finding. In accordance with earlier reports, IGF-I expression was present in the distraction-induced bone regenerate and the osteotomy callus.[12,14,30,51] In the present study, distraction osteogenesis did not result in modulation of IGF-I plasma concentrations during lengthening or the level of IGF-I expression in the consolidation phase. Whether

IGF-I up-regulation in the distraction zone plays an important role during active distraction remains unclear.

IGF-II is a major constituent of both local and systemic growth factors.[3] Knowledge about the role of IGF-II in bone healing and distraction osteogenesis is very limited. IGF-I and IGF-II were reported to be important as local growth factors for osteoblast survival and apoptosis and to modulate osteoblast-osteoclast interactions critical in bone remodeling.[20,21] In the present study, IGF-II plasma concentrations and the minimal expression of IGF-II in the bone regenerate during consolidation did not supply us with clues on the role of IGF-II.

As IGFBP-3 and IGFBP-5 are considered to stimulate osteoblast function, these IGFBPs are of major interest during distraction osteogenesis. Unfortunately, it has not been possible to determine IGFBP-3 and IGFBP-5 levels in canine plasma. On the other hand, IGFBP-4 is capable of modulating IGF actions in bone.[26] In the present study, IGFBP-4 concentrations were fairly constant and comparable with plasma levels in human individuals.[48] IGFBP-6 preferentially binds and modulates IGF-II.[46,49,53] In the present study, canine plasma IGFBP-6 levels tended to be twice as high as in their human counterparts.[49] The decrease in IGFBP-6 plasma concentrations possibly mediated through TGF-β1 could enhance local availability of IGF-II and thus stimulate bone formation.[16] Due to the lack of proper sequences of canine IGFBP-4 and -6 mRNA's we were not yet able to amplify IGFBP mRNA in canine tissue samples. The systemic and local role of IGFBP-4 and IGFBP-6 remains unclear.

BMP-2, -4, and -6 expression has been demonstrated in distraction osteogenesis in rats.[43] In a rabbit model, BMP-2, -4, and -7 was specifically expressed during active lengthening, whereas BMP expression gradually disappeared in the consolidation phase of the bone regenerate.[40] In the present study, the relatively lower expression of BMP-2 in the distraction-induced bone regenerate during consolidation in comparison with the expression in the osteotomy callus could indicate a more advanced stage of bone formation in the first.

The present study was limited by only evaluating the expression of GH, GHR, IGF-I, IGF-II, and BMP-2 in the consolidation phase of distraction osteogenesis. Determination of gene expression at more than one time point would be ideal to elucidate the role of these factors during active lengthening. In addition, pursuing other osteotropic and angiogenic factors will be essential to further evaluate osteogenesis. Micro-array techniques, in which gene expression profiles of large groups of these factors can be determined simultaneously, look very promising to achieve this goal.

Summarily, up-regulation of GHR expression seems to play an important role in the consolidation phase of distraction osteogenesis. Increased sensitivity to

endogenous systemic GH may promote bone formation and bone maturation mediated through a direct effect of GH.

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