The ternary IGF complex influences postnatal bone acquisition and the skeletal response to intermittent parathyroid hormone

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Abstract

The role of circulating IGF-I in skeletal acquisition and the anabolic response to PTH is not well understood. We generated IGF-I-deficient mice by gene deletions of IGF ternary complex components including: (1) liver-specific deletion of the IGF-I gene (LID), (2) global deletion of the acid-labile (ALS) gene (ALSKO), and (3) both liver IGF-I and ALS inactivated genes (LA). Twelve-week-old male control (CTL), LID, ALSKO, and LA mice were treated with vehicle (VEH) or human PTH(1–34) for 4 weeks. VEH-treated IGF-I-deficient mice (i.e. LID, ALSKO and LA mice) exhibited reduced cortical cross-sectional area ($P=0{\cdot}001$) compared with CTL mice; in contrast, femoral trabecular bone volume fractions (BV/TV) of the IGF-I-deficient mice were consistently greater than CTL ($P<0{\cdot}01$). ALSKO mice exhibited markedly reduced osteoblast number and surface ($P<0{\cdot}05$), as well as mineral apposition rate compared with other IGF-I-deficient and CTL mice. Adherent bone marrow stromal cells, cultured in β-glycerol phosphate and ascorbic acid, showed no strain differences in secreted IGF-I. In response to PTH, there were both compartment- and strain-specific effects. Cortical bone area was increased by PTH in CTL and ALSKO mice, but not in LID or LA mice. In the trabecular compartment, PTH increased femoral and vertebral BV/TV in LID, but not in ALSKO or LA mice. In conclusion, we demonstrated that the presentation of IGF-I as a circulating complex is essential for skeletal remodeling and the anabolic response to PTH. We postulate that the ternary complex itself, rather than IGF-I alone, influences bone acquisition in a compartment-specific manner (i.e. cortical vs trabecular bone).

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Introduction

Insulin-like growth factor-I (IGF-I) is a ubiquitous polypeptide that serves as a regulator of cell growth and function. IGF-I can exert its biological effects using two distinct routes: the endocrine route, i.e. via circulating IGF-I, and the autocrine/paracrine route, which represents locally produced IGF-I (Salmon & Daughaday 1957, LeRoith *et al.* 2001). The endocrine form of IGF-I originates mainly from liver and its synthesis is growth hormone (GH)-dependent. In the circulation, IGFs are bound in a ternary complex of high molecular weight ~150 kDa with a family of high affinity IGF-binding proteins (IGFBPs) and the acid labile subunit (ALS) (Leong *et al.* 1992). Once the ternary complex, which is found primarily in the vascular system, dissociates, the binary complexes of IGFBP–IGFs are removed from the circulation and IGF-I reaches target tissues where it interacts with cell surface receptors. In the skeleton, IGF-I is important for osteoblast differentiation and mineralization, and can stimulate osteoclast recruitment (Mochizuki *et al.* 1992, Zhao *et al.* 2000, Rubin *et al.* 2002, Zhang *et al.* 2002). Serum IGF-I levels correlate positively with bone mineral density (BMD) and

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negatively with fracture risk in humans (Langlois *et al.* 1998, Gamero *et al.* 2000). However, the role of serum IGF-I and its ternary complex as an endocrine modulator of skeletal compartments (i.e. cortical and trabecular) remains controversial.

To study the endocrine and autocrine/paracrine roles of IGF-I in growth and development, we previously generated liver-specific IGF-I gene deleted (LID) mice. In the LID mouse, total circulating IGF-I levels were lowered by 75% and GH levels were correspondingly elevated, approximately fourfold (Yakar *et al.* 1999). Importantly, IGF-I and IGF-II mRNA expression in bone were not altered (Yakar *et al.* 2002*a*, 2002*b*). Growth, as assessed by changes in body weight and body length, did not differ significantly between control (CTL) and LID mice. Skeletal analysis of bones from LID mice using peripheral quantitative computerized tomography (pQCT) revealed a mild reduction in bone size and a marked reduction in femoral volumetric bone density (vBMD) (Yakar *et al.* 2002*a*, 2002*b*). A similar skeletal phenotype was noted following ALS gene inactivation (ALSKO), in which a 65% reduction in circulating IGF-I levels but no increase in GH secretion was observed. A double gene inactivation mouse model of LID/ALSKO (LA) caused an even further reduction in circulating IGF-I levels of 85%-90% and a reciprocal increase in GH levels of about 15-fold (Yakar *et al.* 2002*a*). In contrast to the LID mice, the LA mice showed early post-natal growth retardation, as assessed by body length and femoral length, as well as a significant reduction in femoral vBMD (Yakar *et al.* 2002*a*, 2002*b*). Interestingly, *Igf1* null mice also exhibit a cortical phenotype similar to LA mice, but have increased trabecular bone volume fractions (BV/TV) (Bikle *et al.* 2001, 2002).

Daily administration of parathyroid hormone (human PTH(1–34)) increases bone mass, reduces fracture risk, and is approved for the treatment of severe postmenopausal osteoporosis (Neer *et al.* 2001). PTH principally targets osteoblasts, enhancing osteoblast function, and reducing osteoblast apoptosis (Jilka *et al.* 1999). Recent studies suggest that not all individuals treated with PTH respond in the same manner, implying that local or systemic factors may contribute to the heterogeneity in skeletal responsiveness to this agent (Sellmeyer *et al.* 2004). *In vitro* and *in vivo* studies have shown that a number of osteoblast growth factors are activated by intermittent PTH (Hurley *et al.* 1999, Calvi *et al.* 2003, Qin *et al.* 2003). *In vitro,* PTH induces IGF-I expression and secretion in osteoblasts (McCarthy *et al.* 1989). Additionally, antibodies to IGF-I block PTH-stimulated collagen synthesis in fetal rat calvarial cultures (Canalis *et al.* 1989). Furthermore, intermittent PTH did not stimulate bone formation or increase bone turnover in *Igf1* null mice (Bikle *et al.* 2002). Despite these data, the role of the endocrine IGF regulatory system in mediating the anabolic effects of PTH remains unclear.

To understand the role of the ternary IGF-I complex in bone modeling and the anabolic response to PTH, we studied three strains of IGF-I-deficient mice (i.e. subsequently defined as LID, ALSKO, and LA) as well as CTL mice of the same mixed background with and without PTH treatment. The results of these experiments show that the presentation of IGF-I in the circulation as a ternary complex is essential both for postnatal bone acquisition and for the full anabolic response to PTH.

Materials and Methods

Animal husbandry and experimental design

The generation and genotyping of control (CTL), liver-specific IGF-I deficient (LID), ALSKO and LA mice have been described previously (Yakar *et al.* 2002*a*). These mice were generated on a mixed genetic background of FVB/N, C57Bl/6J and 129Sv. Briefly, ALSKO mice were crossed for 6 generations on C57Bl/6J and than crossed with the LID mice to generate the double gene disrupted mice LID+ALSKO (LA). Crossing of males and females from the F2 generation gave rise to four different genotypes within the same litter: (a) control mice, which are homozygous for the *Igf-1* floxed allele, carry an intact *Als* gene (ALS+/+), and do not carry the Alb-*cre* transgene; (b) LID mice, which are homozygous for the *Igf-1* floxed allele, carry the Alb-*cre* transgene and carry an intact *Als* gene (ALS+/+); (c) ALSKO mice, which are homozygous for the *Igf-1* floxed allele, have two *Als* null alleles (ALS − / −) and do not carry the Alb-*cre* transgene; (d) LID ± ALSKO mice (LA), which are homozygous for the *Igf-1* floxed allele, carry the Alb-*cre* transgene and have two ALS null alleles (ALS − / −). Mice that were heterozygous for any of the alleles *als* or floxed *Igf-1* were not included in the study. No difference was observed between mice that carried one or two copies of the Cre transgene. The above four genotypes represent the mice that were used throughout this study.

To study the effects of intermittent PTH treatment on bone remodeling, twelve-week-old male mice were injected subcutaneously 5 days a week with 50 μg/kg/day human PTH(1–34) (Bachem, Torrance, CA, USA) or vehicle (2% normal mouse serum in saline) over a course of 4 weeks. Calcein (15 mg/kg) was injected intraperitoneally 7 and 2 days before death. Mice were killed at the end of week 4, serum was collected, and the L5 vertebral body and femur were assessed by micro-computed tomography (μCT) and histomorphometry. All procedures were approved by the Animal Care and Use Committee of the National Institute of Diabetes and Digestive and Kidney Diseases, National Institute of Health, Bethesda, MD, USA.

Measurement of biochemical parameters

IGF-I Serum IGF-I was measured using an RIA with a polyclonal human anti-IGF-I antibody that has previously

been validated in mice (Rosen *et al.* 2004). Briefly, serum samples were treated with an acid-ethanol preparation removing IGF binding proteins. IGF-II was then added to remove residual or partially proteolyzed IGFBPs, and the remaining sample was then assayed using a double antibody method (Alpco, Windham, NH, USA). The sensitivity of the assay is 0·3 ng/ml and there is no cross reactivity with IGF-II. The interassay coefficient of variation was 4·6%, and the intra-assay coefficient of variation was 2·3%. Known standards are placed in each run, and pooled mouse serum from B6 mice at 16 weeks of age is used as another control for assay variation. IGF-I was also measured in the conditioned media (CM) of bone marrow stromal cells after 14 days - 7 days in 10% fetal calf serum and αMEM, and 7 days of β-glycerol phosphate and ascorbic acid. The same RIA that was used for serum was employed in the CM.

IGFBP-2 Serum IGFBP-2 was measured using an RIA with a polyclonal human antibody to IGFBP-2 (DSL, Webster, TX, USA) validated previously for mouse serum (Rosen *et al.* 2004). The interassay coefficient of variation is 9·6% using C57Bl/6J control mouse sera from 16-week-old males. The sensitivity of the assay is 5 ng/ml and there is no cross-reactivity with IGFBP-1 or IGFBP-3.

Growth hormone (GH) Serum concentrations of GH were determined using an RIA kit with a sensitivity of 0·02 ng/ml (National Hormone and Pituitary Program, Harbor-UCLA Medical Center, Torrance, CA, USA).

Mouse IGFBP-3 and ALS ELISA assays Mouse recombinant IGFBP-3 and ALS standards, rat anti-mouse IGFBP-3 and ALS monoclonal antibodies, and biotinylated goat anti-mouse IGFBP-3 and ALS antibodies were purchased from R&D Systems (Minneapolis, MN, USA). Microtiter plates were obtained from Nalge Nunc International (Rochester, NY, USA). Streptavidin-horseradish peroxidase conjugate, *o*-phenylenediamine dihydrochloride and hydrogen peroxide substrate were purchased from Pierce (Rockford, IL, USA). Microtiter plates (96-well) were coated with capture antibodies at 0·5 g/well in 100 ml phosphate-buffered saline (PBS) (137 mM NaCl, 2·7 mM KCl, 8·1 mM Na_2HPO_4, and 1·5 mM KH_2PO_4, pH 7·4). The plate was incubated overnight at room temperature on a shaker. The antibody-coated plate was washed three times with 300 ml/well of wash buffer (PBS containing 0·05% Tween-20) followed by the addition of 300 ml blocking buffer (PBS containing 5% Tween-20, 5% sucrose, and 0·05% sodium azide) to each well and incubation for an additional 1 h. After washing three times with wash buffer, the plates were used for IGFBP-3 or ALS assays as appropriate. Recombinant mouse IGFBP-3 or ALS standard was diluted in sample dilution buffer in concentrations ranging from 0 to 25 ng/ml for IGFBP-3 and from 0 to 250 ng/ml for ALS. The mouse serum samples were pre-diluted 100- to 400-fold prior to assay. Standards, controls, or diluted samples (100 ml) were added to appropriate wells and incubated at room temperature for 2 h on a shaker. The wells were then washed three times with wash buffer followed by the addition of detection antibody (20 ng/well in 100 ml PBS containing 5% Tween-20 and 2% normal goat serum) and incubated at room temperature for 2 h. The wells were washed three times again and 100 ml streptavidin-horseradish peroxidase conjugate was added to each well and incubated for 20 min at room temperature. After washing four times with wash buffer, 100 ml *o*-phenylenediamine (1 mg/ml in hydrogen peroxide substrate) was added to each well and incubated for an additional 20 min. The reaction was stopped by the addition of 50 ml 1 M sulfuric acid and the absorbance was determined at 490 nm with a plate reader (Molecular Design, Sunnyvale, CA, USA). The IGFBP-3 assay has a sensitivity of 0·2 ng/ml. There is no cross-reactivity with mouse IGFBP-1 or –2, human IGFBP-1, -2, -3 or –4, mouse IGF-I or -II or human IGF-I or -II (all test substances at 100 ng/ml). The intra-assay and interassay coefficients of variation were less than 6% in the range from 1 to 6 ng/ml and recovery was 89 to 104% in the assay range from 2 to10 ng/ml. The mouse ALS assay has a sensitivity of 1·0 ng/ml. There is no cross-reactivity with mouse IGFBP-1, -2 or -3, human IGFBP-1, -2, -3 or –4, mouse IGF-I or -II or human ALS, IGF-I or -II. The intra-assay and interassay coefficients of variation were less than 10% in the range from 10 to 60 ng/ml and recovery was 94 to 102% in the range from 15 to 85 ng/ml.

Osteocalcin Osteocalcin was measured in 10 µl serum by a standard equilibrium radioimmunoassay using specific goat anti-mouse osteocalcin antibody and authentic purified mouse osteocalcin as standard and tracer (Rosen *et al.* 2005). Quality control was routinely monitored in each assay by assaying two known samples that fall in the low and high concentration regions of the standard curves (Rosen *et al.* 2005).

Bone morphology by µCT

Micro-computed tomography (µCT, µCT40; Scanco Medical AG, Basserdorf, Switzerland) was used to assess trabecular bone volume fraction and microarchitecture in the excised 5th lumbar vertebral body and distal femoral metaphysis, and cortical bone geometry at the mid-femoral diaphysis, as previously described (Rosen *et al.* 2005). Morphometric variables were computed from the binarized images using direct, three-dimensional techniques that do not rely on any prior assumptions about the underlying structure (Hildebrand & Ruegsegger 1997, Laib *et al.* 1997). For trabecular bone regions, we assessed the bone volume fraction (BV/TV, %), trabecular

Table 1 Serum parameters for control (CTL), LID, ALSKO and LA mice after 4 weeks of vehicle (VEH) or PTH treatment

	Control (CTL)		**LID**		**ALSKO**		**LA**	
	VEH (n=10)	PTH (n=8)	VEH (n=9)	PTH (n=9)	VEH (n=13)	PTH (n=10)	VEH (n=9)	PTH (n=11)
IGF-I (ng/ml)	249±12	250±16	65±5[a]	72±4	117±8[a]	121±6	41±1[a]	41±1
IGFBP-2 (ng/ml)	411±106	511±129	315±19[b]	339±48	264±40[b]	230±25	219±24[b]	214±26
IGFBP-3 (ng/ml)	294±66	354±93	160±7[a]	126±9	6±1[a]	4·5±1·3	17±1[a]	18±1
ALS (ng/ml)	824±68	NM	5718±307[a]	NM	0[a]	NM	0[a]	NM
GH (ng/ml)	4·1±0·8	NM	9·9±4·6[c]	NM	3·2±0·5	NM	13·4±3·1[c]	NM
Osteocalcin (ng/ml)	71·2±5·0	87·0±11·3	73·8±5·3	147·1±14·3[d]	29·2±3·2[b]	39·6±3·0	54·8±4·8[c]	82·5±4·0[e]

[a]P<0·0001 vs CTL within VEH; [b]P=0·001 vs CTL within VEH; [c]P<0·05 vs CTL within VEH; [d]P<0·001 vs VEH within genotype; [e]P<0·01 vs VEH within genotype. NM, not measured

thickness (TbTh, μm), trabecular number (TbN, mm^{-1}), and trabecular separation (TbSp, μm). For cortical bone at the femoral midshaft, we measured the average total cross-sectional area inside the periosteal envelope (TA, mm^2), the cortical bone and medullary area within this same envelope (BA, mm^2 and MA, mm^2 respectively), the bone area fraction (BA/TA, %), and the average cortical thickness (CortTh, μm).

Bone histomorphometric analysis

Femurs were dissected, fixed in 70% ethanol, dehydrated and embedded undecalcified in methyl methacrylate. Longitudinal sections, 5 μm thick, were cut on a Microm microtome (Microm, Richards-Allan Scientific, Kalamazoo, MI, USA) and stained with Toluidine Blue. Static parameters of bone formation and resorption were measured at a standardized site in the distal femoral metaphysis using an OsteoMeasure morphometry system (Osteometrics, Atlanta, GA, USA). Relative trabecular bone volume (BV/TV), osteoid surface (OS/BS, %), eroded surface (ES/BS, %), osteoblast or osteoclast number per bone perimeter (NOb/BPm, NOc/BPm) and osteoblast or osteoclast number per total area (NOb/Tar, NOc/Tar) were measured. For dynamic histomorphometry, mineralizing surface per bone surface (MS/BS, %) and mineral apposition rate (MAR, μm/day) were measured in unstained 10 μm sections under ultraviolet light, using a B-2A set long pass filter consisting of an excitation filter ranging from 450 to 490 nanometers (nm), a barrier filter at 515 nm, and a dichroic mirror at 500 nm. Bone formation rate per bone surface (BFR, $\mu m^3/\mu m^2$/day) was calculated. The terminology and units used are those recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research (Parfitt *et al.* 1987).

Statistical analysis

Standard descriptive statistics were computed for all variables. Underlying differences in serum biochemistry, bone microarchitecture and histomorphometric variables between different mutants were evaluated within vehicle (VEH)-treated animals by ANOVA, followed when appropriate by *post-hoc* testing using Fisher's Least Squares Protected Difference test. The effect of PTH on these variables was assessed first by two-factor ANOVA, with genotype (CTL, LID, ALSKO and LA) and treatment (PTH or VEH) as independent variables. The effect of PTH within each genotype was assessed by unpaired t-test between VEH- and PTH-treated mice. In all cases, tests were two-tailed, and significance was set at P<0·05, unless otherwise noted. Data are reported as means ± S.E.M.

Results

Serum IGF ternary complex components are altered in IGF-I-deficient mice

To assess how circulating IGF-I presentation affects skeletal parameters, we determined serum levels of IGF-I, IGFBP-2, IGFBP-3, ALS and GH in the various IGF-I-deficient mice (Table 1). Evaluating the vehicle-treated groups, we confirmed that both serum IGF-I and IGFBP-2 concentrations were significantly decreased in ALSKO, LID and LA compared with CTL mice (P<0·001 for all, Table 1). A reduction in serum IGF-I concentrations in LID mice led to a significant reduction in IGFBP-3 levels, possibly due to degradation (data not shown), and when the ALS gene was inactivated (i.e. ALSKO and LA mice), IGFBP-3 concentrations were reduced to almost undetectable levels (Table 1). In the LID and LA mice, the low levels of IGF-I in serum are insufficient to inhibit growth hormone (GH) secretion, and therefore spontaneous GH levels were two- to threefold higher in LA and LID mice compared with CTL (P<0·05 for both). In contrast, ALSKO mice did not exhibit an increase in GH despite a significant reduction in serum IGF-I (Table 1). GH largely controls ALS levels. Therefore, in the LID mice where GH levels were elevated, there was a sevenfold increase in serum ALS compared with CTL mice (P<0·0001). As expected, in ALSKO and LA mice the ALS was undetectable. Serum

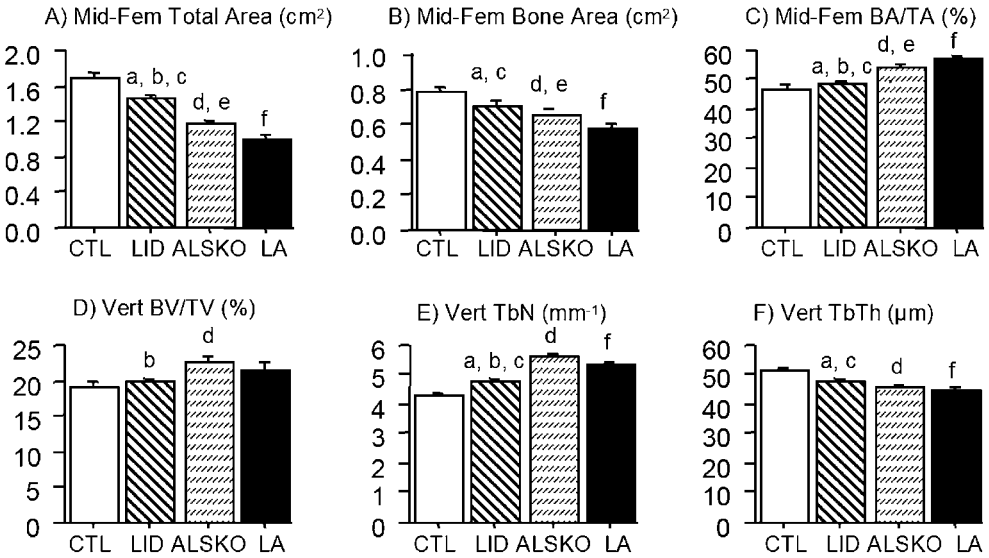

Figure 1 Cortical and trabecular bone microarchitecture, assessed by micro-computed tomography, in VEH-treated control (CTL), LID, ALSKO and LA mice. (A) Mid-femoral total cross-sectional area (cm^2), (B) mid-femoral cortical bone area (cm^2), (C) mid-femoral cortical bone area fraction (BA/TA, %), (D) vertebral trabecular BV/TV (%), (E) vertebral trabecular number (TbN, mm^{-1}), and (F) vertebral trabecular thickness (TbTh, μm). Symbols represent statistically significant differences ($P<0·05$) between (a) LID vs CTL, (b) LID vs ALSKO, (c) LID vs LA, (d) ALSKO vs CTL, (e) ALSKO vs LA and (f) LA vs CTL.

osteocalcin, a bone-specific protein secreted by osteoblasts, was not altered in the LID mice as compared with controls; however, it was markedly depressed in ALSKO mice ($P<0·001$ for ALSKO vs CTL, Table 1). LA mice displayed intermediate levels of osteocalcin, which may have been related to the secondary increase in GH levels.

Alterations in the IGF ternary complex influence skeletal growth and remodeling

We found striking differences in the cortical and trabecular skeleton in VEH-treated IGF-I-deficient mice (Figs 1 and 2). Mid-femoral total cross-sectional area was markedly decreased when circulating IGF-I concentrations were low (CTL>LID>ALSKO>LA; $P<0·001$ for all), and cortical BA/TA displayed a reciprocal increase ($P<0·0001$ for all). Cortical thickness was greatest in ALSKO (208 ± 3 μm) and lowest in LID (194 ± 4 μm, $P=0·02$ vs ALSKO), but otherwise did not differ significantly between genotypes.

Interestingly, a different pattern was observed for trabecular bone structure (Figs 1 and 2). In the vertebral body, trabecular bone volume fraction by μCT was higher in ALSKO and LA compared with CTL ($P=0·002$ and $P=0·06$ respectively). Trabecular number was greater in ALSKO and LA than LID ($P<0·003$), but all groups were greater than CTL ($P<0·007$ for all). Trabecular thickness was lower in IGF-I-deficient mice than in CTL mice ($0·018<P<0·001$). Trabecular bone parameters at the distal femur, assessed by μCT, followed the same pattern as those in the vertebral body (data not shown, see Fig. 2).

To determine how bone modeling is affected by changes in the GH/IGF-I system, we performed histomorphometric measurements of the secondary spongiosa in the distal femur (Table 2). The most striking observation was the marked suppression of osteoblast number ($P<0·02$ vs all other groups), osteoblast surface ($P<0·001$ vs all other groups), and osteoid surface ($P=0·02$ vs CTL) in ALSKO mice. Mineralizing surface and bone formation rates were higher in LID than ALSKO mice ($P=0·01$ for both), but otherwise did not differ among groups. The mineral apposition rate was significantly lower in the ALSKO mice compared with the other IGF-I-deficient groups ($P<0·05$) and marginally lower than CTL mice. Notable was the consistent increase in osteoclastic parameters in LA mice, reflected by greater eroded surfaces ($P=0·01$) and osteoclast surface/bone surface ($P=0·05$) compared with CTL mice. There was also a trend for more osteoclasts per bone perimeter in LA compared with CTL mice ($P=0·08$).

Although we had previously found no expression differences in IGF-I mRNA from the femorae of the various mouse groups, we wanted to exclude differences in IGF-I secretion as a possible cause for the distinct skeletal phenotypes of the IGF-I-deficient mice (Yakar *et al.* 2002*a*). We cultured adherent marrow stromal cells after one week of 10% FCS and αMEM, followed by one week of osteoblast differentiation media. We found no

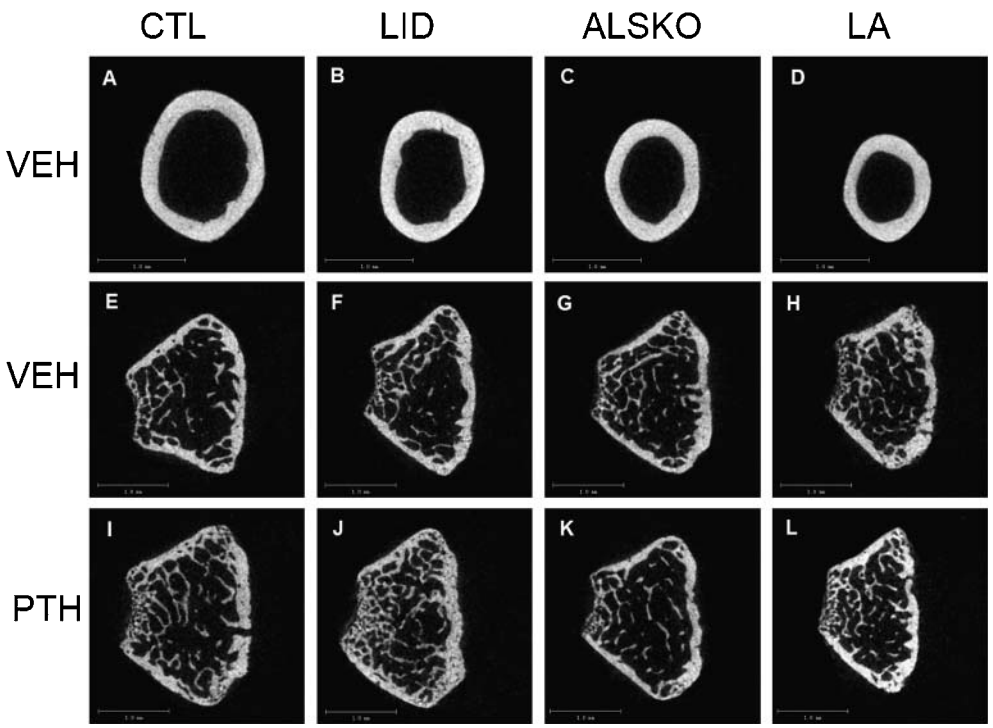

Figure 2 Representative microcomputed tomography images of the femoral midshaft in VEH-treated mice (upper panel), and the distal femoral metaphysis in both VEH- and PTH-treated mice (middle and lower panels).

differences in secreted IGF-I among CTL, LID, ALSKO or LA mice (data not shown). Taken together, these data suggest that in IGF-I-deficient mice without a compensatory increase in GH (i.e. ALSKO mice) or changes in local production of IGF-I, there is significantly decreased osteoblast function.

Alterations in the IGF ternary complex influence skeletal responsiveness to intermittent PTH

PTH treatment did not affect circulatory concentrations of the IGF complex (i.e. IGF-I, IGFBP-2 or IGFBP-3) (Table 1). In contrast, osteocalcin concentrations increased following PTH in all groups, reaching significance in PTH-treated LID and LA mice (+99% vs VEH, $P<0{\cdot}001$ and +50% vs VEH, $P<0{\cdot}01$ respectively). The effects of PTH on cortical and trabecular bone microarchitecture differed by genotype. Cortical bone anabolic responses, assessed at the mid-femur, were strongest in CTL and ALSKO mice, in whom there were significant gains in cortical bone area (+13·7% $P=0{\cdot}042$ and +9·3%, $P=0{\cdot}027$ respectively) and cortical thickness (+6·4%, $P=0{\cdot}09$ and +6·0%, $P=0{\cdot}018$ respectively) (Fig. 3).

In contrast to the effect of PTH on cortical bone, trabecular bone volume fraction increased most dramatically and consistently in LID mice (+26% vs VEH at vertebrae, $P=0{\cdot}002$ and +50% vs VEH at the distal femur, $P=0{\cdot}003$) (Figs 2 and 3). Trabecular number and thickness were also consistently higher in PTH-treated LID compared with VEH. In contrast, trabecular bone volume was unchanged in ALSKO at both skeletal sites and unchanged in LA at the vertebrae, but declined significantly at the distal femur (−27% vs VEH, $P=0{\cdot}05$) (Figs 2 and 3). In summary, the μCT data show that in the trabecular bone compartment, LID mice had a consistent anabolic response, whereas ALSKO and LA mice either had no change or a catabolic response to intermittent PTH treatment.

The effects of PTH on trabecular architecture assessed by histomorphometry were similar to those detected by μCT, with a marked increase in BV/TV in LID, no change in CTL and ALSKO, and a significant decline in LA mice (Table 2 and Fig. 2). Interestingly, despite variable changes in trabecular bone volume among the different genotypes, PTH treatment led to consistent increases in osteoblast number and bone formation rate. The effects of PTH on osteoclasts varied significantly by genotype ($P<0{\cdot}007$). CTL mice responded to PTH with an increase in osteoclast number and eroded surfaces ($0{\cdot}06<P<0{\cdot}01$). LID and ALSKO showed a tendency for increases in osteoclastic parameters, whereas LA did not (Table 2). Taken together, these data suggest that, even though the mechanisms responsible for PTH anabolic actions on the skeleton are not clearly defined, changes in

Table 2 Histomorphometric analysis of the distal femur (mean ± S.E.M.) in vehicle (VEH) and PTH-treated control, LID, ALSKO and LA mice

	Control (CTL)		**LID**		**ALSKO**		**LA**		
	VEH (*n*=6)	PTH (*n*=6)	VEH (*n*=6)	PTH (*n*=6)	VEH (*n*=6)	PTH (*n*=6)	VEH (*n*=6)	PTH (*n*=5)	$P_{genotype}$
BV/TV (%)	12·3 ± 0·6	12·3 ± 2·1	10·6 ± 0·4	21·0 ± 0·8**	12·3 ± 0·5	10·4 ± 1·0	14·3 ± 1·2	7·6 ± 0·3	<0·001
OS/BS (%)	4·01 ± 1·40	3·08 ± 0·71	2·02 ± 0·49	5·95 ± 1·17[e]	0·90 ± 0·27[a]	3·13 ± 0·60[e]	2·52 ± 0·43	4·34 ± 0·56[e]	ns
ES/BS (%)	17·8 ± 2·6	27·7 ± 2·0[e]	23·3 ± 1·8	27·6 ± 1·5	22·7 ± 2·3	27·9 ± 1·9	26·0 ± 1·8[a]	25·9 ± 3·7	ns
NOb/BPm (mm^{-1})	31·2 ± 3·0	56·0 ± 6·5[f]	25·2 ± 2·9	53·1 ± 6·3[f]	12·8 ± 2·1[a,b,d]	37·8 ± 4·1[f]	24·1 ± 3·5	56·0 ± 6·2[f]	0·0013
NOb/Tar (mm^{-2})	406 ± 42	689 ± 93[e]	324 ± 39	960 ± 132[f]	183 ± 29[a,b,d]	523 ± 69[f]	333 ± 47	522 ± 64[e]	0·0014
ObS/BS (%)	18·5 ± 1·7	32·6 ± 3·0[f]	15·6 ± 1·4	33·0 ± 3·7[f]	8·3 ± 1·5[a,b,d]	26·3 ± 2·8[f]	15·1 ± 2·0	34·5 ± 3·8	0·0084
NOc/BPm (mm^{-1})	5·1 ± 0·7	7·7 ± 0·7[e]	5·8 ± 0·4	7·0 ± 0·6	6·3 ± 0·8	8·6 ± 0·7[e]	6·7 ± 0·5	7·8 ± 1·7	ns
NOc/Tar (mm^{-2})	66 ± 9	93 ± 9[e]	74 ± 5	126 ± 10[f]	90 ± 11	106 ± 13	95 ± 11[a]	70 ± 11	0·007
Ocs/BS (%)	5·3 ± 0·7	8·2 ± 0·7[e]	6·5 ± 0·4	7·7 ± 0·7	7·0 ± 1·0	9·3 ± 0·7	7·3 ± 0·6[a]	8·5 ± 1·8	ns
MS/BS (%)	4·75 ± 0·76	6·70 ± 0·78	6·69 ± 0·83	8·49 ± 1·46	3·48 ± 0·89[b]	10·10 ± 0·98[e]	4·85 ± 0·83	7·55 ± 1·33	ns
MAR (μm/day)	0·37 ± 0·04	0·58 ± 0·06[e]	0·43 ± 0·03	0·63 ± 0·02[e]	0·34 ± 0·02[d]	0·63 ± 0·02[f]	0·45 ± 0·04	0·65 ± 0·09[e]	ns
BFR ($\mu m^3/\mu m^2$/day)	0·018 ± 0·004	0·039 ± 0·005[e]	0·030 ± 0·005	0·055 ± 0·014	0·012 ± 0·004[b]	0·063 ± 0·006[f]	0·022 ± 0·004	0·050 ± 0·014	ns

[a]P<0·05 vs CTL within VEH groups; [b]P<0·05 vs LID within VEH; [c]P<0·05 vs ALSKO within VEH group, [d]P<0·05 vs LA within VEH group; [e,f]P<0·05, P<0·01 vs VEH within genotype; ns, not significant.
BV/TV, trabecular bone volume fraction; OS/BS, percent osteoblast surface per bone surface; ES/BS, percent eroded surface per bone surface; NOb/BPm-number of osteoblasts per bone perimeter; NOb/Tar, NOb per total area; Obs/BS, osteoblasts per bone surface; NOc/BPm, number osteoclasts/bone perimeter; NOc/Tar, number of osteoclasts per total area; OcS/BS, osteoclasts per bone surface; MS/BS, percent mineral surface/bone surface; MAR, mineral apposition rates; BFR, bone formation rate.

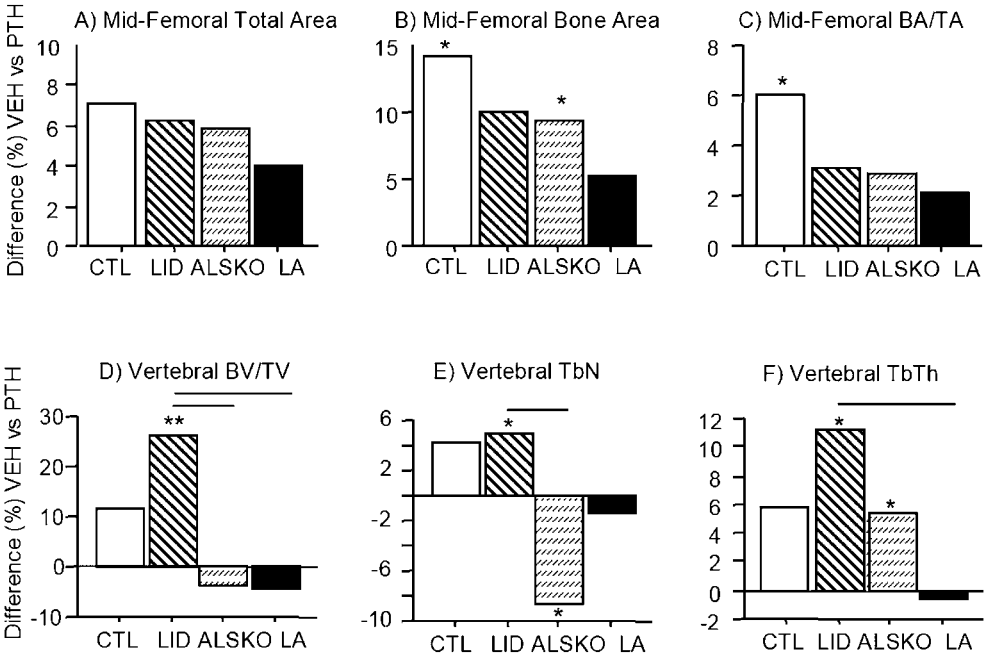

Figure 3 Mean percentage difference in cortical and trabecular bone microarchitecture, assessed by micro-computed tomography, between VEH- and PTH-treated control (CTL), LID, ALSKO and LA mice. (A) Mid-femoral total cross-sectional area, (B) mid-femoral cortical bone area, (C) mid-femoral cortical bone area fraction, (D) vertebral trabecular BV/TV, (E) vertebral trabecular number (TbN), and (F) vertebral trabecular thickness (TbTh). *P<0·05, **P<0·01 for PTH-treated vs VEH-treated. Bars indicate significant (P<0·01) differences between groups in the response to PTH.

the presentation of circulating IGF-I (i.e. ternary vs binary complexes) influence the cellular response to intermittent PTH treatment.

Discussion

In this study we used genetically altered mice (i.e. IGF-I-deficient mice) to examine the influence of the IGF ternary complex on postnatal bone acquisition, as well as the skeletal response to intermittent PTH. Our findings support the thesis that components of the ternary complex (i.e. IGF-I, IGFBP-3 and ALS) modulate bone acquisition and remodeling through compartment-specific mechanisms (Fig. 4). First, we showed that the cortical skeleton was profoundly affected by changes in complex components of VEH-treated IGF-I-deficient mice. For example, LID had a 15% decrease, ALSKO had a 30% decrease, and LA had a 40% decrease in mid-femoral cross-sectional area compared with CTL mice. But these skeletal changes were not reliably predicted by serum IGF-I concentrations. For example, ALSKO mice exhibited smaller periosteal circumferences and less cortical bone area, but actually had higher serum IGF-I levels than LID mice.

In the absence of the ALS, a reduction in circulating IGFBP-3 limits the ability to generate the ternary complex (see Fig. 4). This would lead to a shorter half-life of IGF-I in the circulation, since the unbound IGF-I would be degraded more rapidly. In turn, this could result in less bioactive IGF-I at the tissue level. Alternatively, it is conceivable that the ALS has a direct effect on bone remodeling in addition to its role in binding to IGFBP-3. However, ALS is not expressed in bone or bone marrow stromal cells, hence its effect must be explained by endocrine mechanisms (Ahlborg *et al.* 2003) (Novartis, http://symatlas.gnf.org/SymAtlas/).

In sharp contrast to cortical bone changes, the trabecular compartment of the distal femur and vertebrae revealed a different pattern. By μCT, ALSKO and LA mice had the highest trabecular BV/TV and the greatest number of trabeculae, albeit thinner than CTL mice. The skeletal structure of LA and ALSKO resemble *Igf1* null mice with increased trabecular BV/TV (Bikle *et al.* 2001, Miyakoshi *et al.* 2001, Bouxsein *et al.* 2005). Although the mechanism(s) responsible for this increase in *Igf1* null mice is not known, two hypotheses have emerged: (1) there is an absence of bone resorption due to a lack of skeletal IGF-I, or (2) there is a marked increase in GH secretion as a compensation for low circulating IGF-I. With respect to the former scenario, in our current study the number of osteoclasts per bone surface and the eroded surface/bone surface, a measure of active bone resorption, in VEH-treated ALSKO and LA mice were equal to or

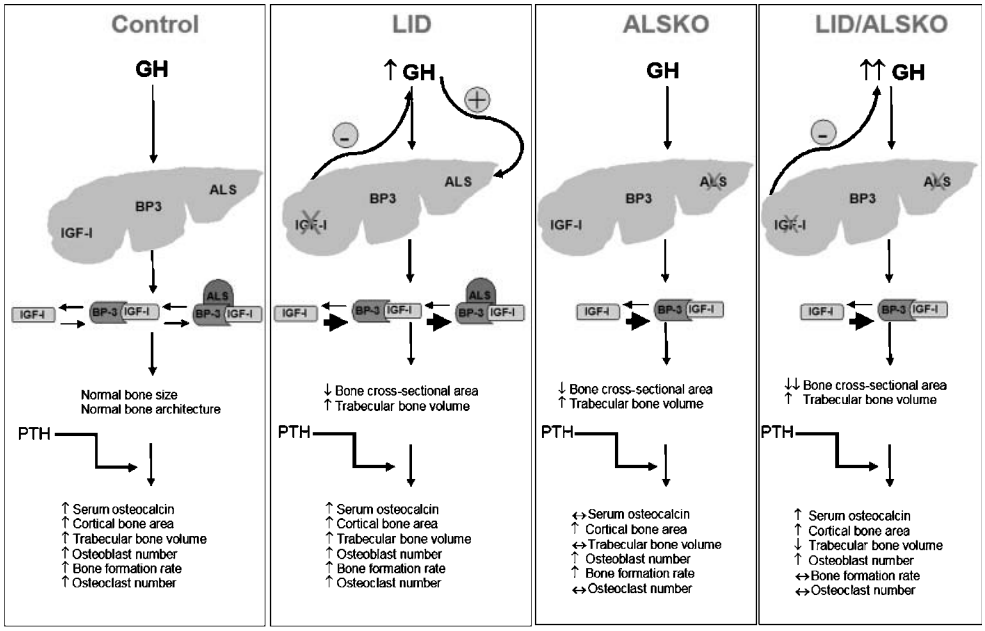

Figure 4 Summary of the skeletal effects resulting from alterations in the IGF-I ternary complex. BP3, binding protein 3.

higher than CTL or LID mice. This would exclude the possibility that ALSKO and LA mice have high trabecular BV/TV because of less bone resorption at 16 weeks of age. Also, we did not find altered skeletal IGF-I expression in any of the IGF-I-deficient mice, nor in their stromal cells.

The second thesis that a compensatory increase in GH produces higher trabecular BV/TV deserves closer scrutiny. Another genetically engineered mouse, the insulin receptor substrate (*IRS1*) null mouse provides some additional insight into this question, since it did not have an anabolic response to PTH despite normal circulating IGF-I concentrations (Yamaguchi *et al.* 2005). *IRS1* null mice have low cortical and trabecular bone mass without a compensatory increase in GH, implying that a rise in GH secretion is essential for maintenance of trabecular bone mass in mice with reduced IGF-I signaling (Yamaguchi *et al.* 2005). But, ALSKO mice have higher trabecular bone volume fractions than CTL mice, and yet do not show a secondary rise in GH, suggesting another compensatory mechanism could be operative, such as enhanced responsiveness to mechanical loading. However, a limitation in comparing compensatory responses to gene deletion is the genetic background of the lines studied. *IRS1* null mice were created on a B6/CBA F1 background, *Igf1*-/- mice were generated in a CD-1 background and the mice reported in this study were on a mixed background (B6/129/FVBN) (Bikle *et al.* 2001, Yamaguchi *et al.* 2005). Hence, further studies will be needed to delineate the functional significance of this paradoxical increase in trabecular bone volume fraction among ALSKO and LA mice.

Another line of evidence that supports a role for the ternary complex in bone turnover is the striking reduction in several parameters related to osteoblast activity in ALSKO mice, without differences in pre-osteoblast IGF-I secretion. This would argue against a deficiency in skeletal IGF-I as the major mechanism responsible for this striking phenotype. On the other hand, LA mice demonstrated significantly greater indices of osteoblast activity (i.e. serum osteocalcin, bone formation rate, mineral apposition rate, and number of osteoblasts per bone perimeter) than ALSKO mice, likely as a result of higher GH concentrations. Overall, the highest rates of bone formation, and the greatest skeletal response to PTH were found in LID mice, who also had the highest ALS concentrations and increased GH (Table 1). Taken together, these data indicate that the ALS plays a vital role in the process of bone formation. That tenet is reinforced by a recent report of an individual with a mutation in the ALS gene resulting in the absence of the circulating IGF ternary complex, and very low areal bone mineral density as measured by dual x-ray absorptiometry (DXA) (Domene *et al.* 2004).

Previous studies demonstrated that skeletal IGF-I was critical for the full anabolic effect of PTH and for bone mineralization (Bikle *et al.* 2001, Miyakoshi *et al.* 2001, Zhang *et al.* 2002). The most compelling evidence that the ternary complex is also important for optimal responsiveness to PTH is derived from our current study.

Although PTH modestly increased cortical bone area in all experimental groups, we found the trabecular response at both the vertebrae and distal femur was markedly influenced by genotype. For example, the greatest increase in trabecular bone volume was in LID mice, while ALSKO showed no change and LA mice exhibited a 'catabolic' response to PTH, i.e. a marked loss in trabecular BV/TV. These changes occurred despite a PTH-induced rise in osteoblast number, mineral apposition rate, and bone formation rate in all groups. Conversely, osteoclast activity was only enhanced in those mice that showed an increase in trabecular BV/TV, i.e. wild-type and LID mice.

The sharp contrast in skeletal responsiveness to PTH in the experimental groups is particularly interesting. For example, the 'non-responders' to PTH shared the same common gene deletion of the ALS, and both mutant strains showed a significant impairment in PTH-induced osteoclastic activity. This observation implies that activation of osteoclasts as well as osteoblasts is necessary for a full anabolic response to PTH. This thesis has gained support recently from both mathematical modeling and clinical studies (Black *et al.* 2003, Finkelstein *et al.* 2003, Ettinger *et al.* 2004). But the mechanism responsible for this coupling effect is not clear, although it is conceivable that the defect lies in the signaling between osteoblasts and osteoclasts. One major factor in that process is RANKL, a cytokine produced by stromal cells to induce recruitment of osteoclasts in response to various local and/or endocrine mediators (Lee & Lorenzo 1999). Interestingly, IGF-I is a potent signal for RANKL activation in marrow stromal cells, suggesting that during states of relative IGF-I insufficiency, osteoclast recruitment by a variety of stimuli may be impaired (Rubin *et al.* 2002). Whether the absence of the ternary complex in the marrow milieu, or the amount of 'free' IGF-I, reduces RANKL signaling to osteoclasts, particularly with PTH, will require further testing.

In this study we analyzed male littermates on a mixed background, which more accurately reflects genetic heterogeneity in the human population. However, we cannot exclude the possibility that the effects of such a mixed genetic background would differentially affect skeletal acquisition and the anabolic response to PTH. Similarly, there may be gender specificity in terms of the compartmental response to PTH in mice with specific gene deletions. Notwithstanding those limitations, we have established an essential role for the circulating IGF ternary complex in mediating bone acquisition in the postnatal time period, and the skeletal response to PTH. It is likely that the absence of ALS has a profound effect on the half-life and presentation of IGF-I in the circulation, leading to changes in bone formation and impaired PTH responsiveness. Now that anabolic therapy with PTH is widely available for treating osteoporosis, it would be valuable to determine whether variable presentation of the components of the ternary IGF-I complex in humans could be used to predict skeletal responsiveness to PTH.

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